NKT cells between innate and acquired immunity: function and specificity

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Abbreviations

Ab	antibody
αGalCer	alpha-galactosyl-ceramide
αGalA	alpha-galactosidase A
Ag	antigen
APC	antigen presenting cell
-APC	allophycocy, fluorescent dye for FACS analysis
β2m	beta-2-microglobulin
BCG	bacillus Calmette et Guérin
BCR	B cell receptor
BMMϕ	bone marrow macrophage
BSA	bovine serum albumin
C57BL/6	C57BL/6 = widely used inbred mouse strain.
CD#	"cluster of differentiation": international nomenclature for cell surface molecules (CD number)
CD1d tetramer	tetrameric complex of murine soluble CD1d molecules used for FACS analysis of NKT cells
ConA	concanavalin A
CTL	cytolytic (cytotoxic) T lymphocyte
Cy5	Cy-Chrome 5, fluorescent dye for flow cytometry and microarray analysis
Cy3	Cy-Chrome 3, fluorescent dye for flow cytometry and microarray analysis
Da	Dalton
DAPI	4, 6-diamidino-2-pheylindole (dihydrochloride)
DC	dendritic cells
DMSO	dimethyl sulfoxide
DN	double negative
DNA	deoxyribonucleic acid
DP	double positive
ELISA	enzyme linked immunosorbent assay
ER	endoplasmic reticulum
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FITC	fluoresceinisothiocyanat, fluorescent dye for FACS analysis
GM-CSF	granulocyte / macrophage colony-stimulating factor
IFNγ	interferon γ
Ig	immunoglobulin
iGb3	iso-globo-trihexosylceramide
IL	interleukin
i.v.	intravenous
i.p.	intraperitoneal
Gb3	globo-trihexosylceramide
HPLC	high pressare liquid chromatography
kDa	kilodalton
LAM	lipoarabinomannan

Lamp1	lysosomal-associated membrane protein 1
Li	invariant chain
LM	lipomannan
LPS	lipopolysaccharid
mAb	monoclonal antibody
M	mol per liter
MIIC	MHC class II containing compartment
M. tuberculosis	Mycobacterium tuberculosis
MALDI-MS	matrix assisted laser disorption ionisation - mass spectrometry
MHC	major histocompatibility complex
MOI	multiplicity of infection
MR	mannose receptor
NF-κB	nuclear factor - kappa B
NK	natural killer
NKT cell	natural killer T cell
PBS	phosphate buffered saline
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
PE	phycoerythrin, fluorescent dye for FACS analysis
PE-Cy7	phycerythrin-Cy-Chrome 7 conjugate, fluorescent dye for FACS analysis
PI	propidium iodide
PIM	phosphatidylinositol mannoside
POD	peroxidase
RNA	ribonucleic acid
RT	room temperature
RT-PCR	reverse transcription polymerase chain reaction
s.c.	subcutaneous
sCD1d	soluble CD1d
SDS	sodiumdodecysulphonate, common detergent
TAP	transporter associated with antigen presentation
TCR	T cell receptor
Th1	T helper cell type 1
Th2	T helper cell type 2
TLC	thin layer chromatography
TLR	toll-like receptor
TNF	tumour necrosis factor
Treg cell	regulatory T cell

1 Introduction

The environment contains a huge variety of pathogenic microbes - viruses, bacteria, fungi, protozoa and multicellular parasites. These can cause disease, and if unchecked they eventually kill the host. The immune system has developed to combat infectious agents, to protect the host from these pathogens and to avoid permanent damage. Moreover, it monitors the cells of the host to recognize mutant cells and to avoid tumour development. Because microorganisms come in many different forms, a wide variety of immune responses are required to deal with each type. The immune systems developed multiple protective mechanisms consisting of soluble factors and cells with distinct roles in defence against infections. The site of the infection and the type of pathogen largely determines which immune response will be active.

Any immune response involves firstly, recognition of the pathogen or other foreign, hazardous material, and secondly a reaction to neutralize or eliminate it. The coordinated cooperation of soluble mediators and cells leads to an adequate immune response by initiating inflammation and elimination of the pathogen or mutant cell.
The different immune responses can be classified into two categories: innate immune responses and adaptive immune responses. The important difference between these two mechanisms is that an adaptive immune response is highly specific for a particular pathogen whereas innate immunity recognizes broad, conserved structures of pathogens. Innate responses are not altered on repeated exposure to one infectious agent whereas the adaptive response improves with each successive encounter with the same pathogen leading to immunological memory. A key component to establish and coordinate immune responses are cytokines. These are soluble factors released by cells endowed with the property to initiate, modulate and regulate immune responses.

NKT cells are specialized T cells equipped with innate immunity-like and adaptive immunity-like properties. They are thought to function at the interface of innate and adaptive immunity and are involved in a wide range of immune responses like antitumour responses, host defence to infections and in inflammation. A key factor and unique property of NKT cells is the concomitant production of Th1 and Th2 cytokines.

1.1 Cells comprising the immune system

Immune responses are mediated by a variety of specialized cells with diverse funtions and by soluble molecules which they secrete. These cells arise from a single progenitor, the pluripotent hematopoietic stem cell in the bone marrow. These pluripotent cells divide into two types of progenitor stem cells; the common lymphoid progenitor and the myeloid progenitor cell. The lymphoid progenitor cell gives rise to natural killer (NK) cells and T lymphocytes (T cells) and B lymphocytes (B cells). Unlike T and B cells, NK cells lack antigen specificity. The common myeloid progenitor cell gives rise to macrophages, neutrophils, dendritic cells (DCs), basophils / mast cells, eosinophils and red blood cells. The number and localization of myeloid cells is dynamic and largely depends on the occurence of an immune response and the cytokines and chemokines produced thereby. Myeloid immune cells circulate in the blood and enter tissues only at sites of infection or inflammation. Neutrophil numbers are massively increased during infection and are recruited to sites of infection and inflammation to phagocytose bacteria. Immature Monocytes also enter tissues where they differentiate into macrophages. Macrophages are found in all organs and tissues. Depending on the tissue they reside in, they can further differentiate into specific macrophage like cells, for instance Kupfer cells in the liver and Langerhans cells in the skin. Immature DCs travel through the blood to enter peripheral tissues. Upon encounter with a potential antigen, they mature and migrate to lymphoid tissues where they activate antigen specific T cells. NK cells are important for eradication of pathogen-infected cells, especially during the early phase of infection (Biron et al., 1999). NK cells recognize cell surface changes that occur on a variety of virally infected cells and tumour cells and are believed to be involved in tumour surveillance (Diefenbach and Raulet, 2002).

B cells combat extracellular pathogens in an antigen specific way. Having recognized its specific antigen, B cells multiply and differentiate into plasma cells which produce large amounts of antibodies. T cells can be characterized by the cell surface receptors they express. The majority of T cells express a T cell receptor (TCR) composed of an α and β chain (α/β T cells). A minor group of T cells displays a TCR composed of an γ and δ chain (γ/δ T cells). γ/δ T cells seem to be important in immune responses to bacteria, however their role in adaptive immunity remains unclear (Chen and Letvin, 2003; Kaufmann, 1996). T cells can be further characterized by the specific expression of the coreceptors CD4 and/or CD8. $CD4^+$ and $CD8^+$ T cells differ fundamentally from each other and perform distinct regulatory and effector functions. T and B cells are located in the central lymphoid organs, the thymus (T cells) and the bone marrow (B cells). It is in these compartments where they develop and differentiate. Mature T and B cells can be detected in the peripheral lymphoid organs (e.g.

spleen, lymph nodes, Peyer's patches). Here, they develop further into effector cells. During infection, effector T cells are found notably at the site of inflammation. Cells of the lympoid and myeloid lineage interact and function in an orchestrated fashion to establish a successful immune response. These immune responses can be divided into innate and adaptive immune responses

1.2 Innate immunity

Innate immune responses are initiated instantaneously after recognition of foreign antigens. It is the first and rapid, although relatively unspecific, response of the immune system to an invading pathogen. Innate immune responses are essential to mount an effective immune response and crucial for establishing an adaptive immune response.

1.2.1 Cells of the innate immune system

Activation of innate immunity is initiated by recognition of antigenic, non-self structures by receptors expressed on immune cells. Macrophages located in the submucosal tissue are usually the first cells to encounter pathogens. They are supported by massive infiltration of neutrophils. Macrophages and neutrophils are specialized in phagocytosis and subsequent killing of the invading pathogens. Macrophages and neutrophils recognize pathogens by means of their cell surface receptors, such as mannose receptors or scavenger receptors, which enable them to discriminate between "self" and "non-self" structures (Janeway, Jr. and Medzhitov, 2002). Furthermore, they express receptors for antibody and complement, which increases phagocytosis of antibody- or complement-coated microbes.

NK cells are innate immune cells that mediate cellular cytotoxicity and produce chemokines and inflammatory cytokines such as IFNγ and TNFα (Trinchieri, 1989; Medzhitov and Janeway, Jr., 1997). NK cells are usually constitutively active and shortly after the initiation of infection, NK cells undergo "clonal expansion" by proliferation. The resulting activated NK cells persist over a period of a few days (Dokun et al., 2001). Although the expanded NK cell populations may help clear the virus, they do not, unlike T cells, provide long-lasting NK cell-mediated memory. NK cells also participate directly in adaptive immune responses, by interacting with DCs and by triggering T cell responses. For instance, induction of DC maturation to produce TNFα and IL-12 and upregulation of co-stimulatory ligands is triggered by NK cells (Gerosa et al., 2002). Moreover, NK cells proliferate and acquire cytotoxic activity and the capacity to produce IFNγ through the interaction with DCs (Fernandez et al., 1999). Apart from activation of other cells of innate immunity, NK cells also enhance induction of $CD8^+$ T cell responses (Mocikat et al., 2003). T cell responses are influenced by IFNγ derived

from NK cells, which promotes antigen processing and presentation to T cells and T helper type 1 (Th1) cell polarization.

DCs are, together with B cells and macrophages, professional antigen presenting cells (APCs) and are thought to be key players in initiating an adaptive immune response (Hanada et al., 2005). Immature DCs are specialized in antigen capture and processing. Upon antigen update, DCs mature, display large amounts of major histocompatibility complex (MHC)–peptide complexes at their surface, upregulate their co-stimulatory molecules and migrate to lymphoid organs where they liaise with and activate antigen-specific T cells. DCs are the most potent antigen presenting cells and their activities can be induced by infectious agents and inflammatory products, so that DCs are mobile sentinels that bring antigens to T cells and express co-stimulators for the induction of immunity (Banchereau and Steinman, 1998).

1.2.2 Receptors for innate immune response

Microorganisms display repeating patterns of molecular structure on their surface as well in their nucleic acids. Bacterial DNA, for example, contains unmethylated repeats of the dinucleotide CpG. The innate immune system recognizes such pathogens by means of pattern recognition receptors (PRRs) that bind to features of these regular patterns. This strategy is based on the detection of a limited set of conserved molecular patterns (pathogen-associated molecular patterns (PAMPs)) that are unique to the microbial world and invariant among entire classes of pathogens (Janeway, Jr. and Medzhitov, 1998).
Innate recognition of PAMPs through Toll-like receptors (TLRs) initiates an inflammatory response characterized by the recruitment of cells to sites of infection which augments the killing of invading pathogens. Activation of TLRs induces the expression of genes encoding chemokines, chemokine receptors and integrins that regulate cell migration to the sites of inflammation and initiate responses to inflammation (Huang et al., 2001; Mantovani, 1999).
Stimulation of TLRs through pathogen products also induces the activation of macrophages and DCs and leads to the induction of several important mediators of innate immunity, such as cytokines (Kamath et al., 2005; Pierer et al., 2004). Additionally, surface expression of co-stimulatory molecules like B7.1 (CD80) and B7.2 (CD86) on macrophages and DCs is evoked by the Toll pathway. Along with antigen presentation by MHC class II proteins on DCs and macrophages, these molecules activate $CD4^+$ T cells and are needed to establish an adaptive immune response.
Most mammalian species have 10 to 15 TLRs which detect multiple PAMPs (Takeda et al., 2003), including lipopolysaccharide (LPS) (detected by TLR4), bacterial lipoproteins and lipoteichoic acids (TLR2), flagellin (TLR5), the unmethylated CpG DNA of bacteria and viruses (TLR9), double-stranded RNA (TLR3) and single-stranded viral RNA (TLR7) (Heil et

al., 2004). TLRs 1, 2, 4, 5 and 6 seem to specialize in the recognition of mainly bacterial products that are not made by the host. Especially, TLR2 detects bacterial cell wall components like peptidoglycan, lipoteichoic acid and lipoarabinomannan of gram$^+$ bacteria and mycobacteria (Akira and Takeda, 2004). TLRs 3, 7, 8 and 9, in contrast, specialize in viral detection, are localized to intracellular compartments (Heil et al., 2003) and detect viral nucleic acids in late endosomes-lysosomes. Pattern recognition receptors activate conserved host defence signalling pathways that control the immune responses in a wide variety of cells.

1.3 Linking innate and adaptive immunity

Adaptive immunity commences with DCs capturing microbial antigens in the peripheral tissues. Subsequently, DCs migrate to the draining lymph nodes to present the processed antigens to naive T lymphocytes in the context of antigen presenting molecules such as MHC molecules and CD1 molecules. During transit, DCs undergo a maturation program that provides the cells with the ability to stimulate naive T lymphocytes. Inside the lymph nodes, DCs contact antigen-specific T cells and induce their activation and differentiation into effector cells. T cell activation is achieved by co-presentation of MHC class II molecules with co-stimulatory receptors and the recognition of this complex by the appropriate TCR. The main pathway by which DCs become activated and mature to provide the second signal to naive T cells occurs via the TLR recognition of PAMPs (Medzhitov, 2001). Induction of the co-stimulatory molecules CD80 and CD86 on the DC surface is coupling microbial recognition with the induction of co-stimulators that allows activation of pathogen-specific T cells. Since T cells receive the antigen-specific activation signal only in the context of a co-stimulatory signal, TLR-induced expression of co-stimulators transforms the pattern recognition signal into antigen-specific immune responses.

1.4 The adaptive immune system

In contrast to innate immune responses, adaptive immunity is characterized by two hallmarks: specificity and memory.

1.4.1 Cells of the adaptive immune response

The adaptive immune response is induced by activation of B and T cells leading to clonal expansion of B and T cells following recognition of their cognate antigen. B cells recognize soluble antigens via the B-cell receptor (BCR), a membrane bound form of antibody. Upon antigen recognition B cells start to proliferate and differentiate into IgM secreting effector

cells, the plasma cells. In a T-cell dependent process, a subpopulation of activated B cells undergoes differentiation to produce other antibody isotypes, a process called isotype switch (Oettgen, 2000).

T cell activation is achieved by co-presentation of the MHC/antigen complex with co-stimulatory receptors and the recognition of this complex by the appropriate TCR/CD3 complex. Co-stimulatory receptors expressed on DCs trigger CD28 expressed on naive T cells and activation leads to clonal expansion and the generation of effector T cells. A fraction of the activated B and T cells matures to long-living memory B and T cells providing the basis for a fast and highly specific response to further infection with antigen.

1.4.2 Receptors for adaptive immune responses

The TCR and BCR are, unlike the receptors of innate immunity, highly specific for their cognate antigen. They are generated in immature B and T cells by recombination of different germline gene segments. This process provides the means for the generation of a highly diverse pool of T and B cells, each expression a unique TCR or BCR. To avoid the generation of TCRs and BCRs that recognize structures of the host, developing T and B cells are eliminated by negative selection in case their TCR or BCR recognizes host derived structures. CD8 and CD4 molecules are coreceptors expressed on the surface of T cells and bind to MHC class I or MHC class II molecules, respectively. They support MHC molecule recognition by the TCR/CD3 complex and increase the sensitivity of antigen recognition by the T cell (Weiss et al., 1991).

1.4.3 Different T cell populations produce different adaptive responses

Depending on the types of co-stimulatory molecules expressed and cytokines secreted by the DCs, naive $CD4^+$ T cells differentiate into either T helper 1 (Th1) or T helper 2 (Th2) cells (Constant and Bottomly, 1997). While the presence of IL-12 promotes the development of Th1 cells, IL-4 induces the development of Th2 cells. Th1 and Th2 cells secrete different types of cytokines and thereby mediate different types of adaptive immune responses (Farrar et al., 2002). Th1 cells produce IFNγ, TNFα, IL-2 and lymphotoxin. These pro-inflammatory cytokines augment the microbicidal capacity of monocytes and macrophages, mediate the development of cytotoxic $CD8^+$ T cells and are important in establishing inflammation. Th2 cells produce IL-4, IL-5, IL-10 and IL-13. Biological functions of these cytokines are activation of B cell, regulation of IgE secretion by B cells, modulation of TH2 cell development and suppression of inflammatory responses (McKenzie and Heath, 1996) (Figure 1).

$CD8^+$ T cells are cytotoxic effector T cells (CTL) and essential for the host defense against intracellular pathogens, like viruses and bacteria like listeria and mycobacteria (Kaufmann et al., 1995; Mittrucker and Kaufmann, 2000). CTLs identify infected cells by the recognition of

foreign peptides presented by major histocompatibility complex (MHC) class I molecules on the surface of virtually every cell of the host. CTLs attack and destroy cells infected with cytosolic pathogens by various mechanisms. In addition, they also produce IFNγ which inhibits viral replication and induces increased MHC class I presentation and macrophage activation (Chan et al., 1991b). Primary activation of $CD8^+$ T cells requires costimulatory signals from DCs, whereas memory $CD8^+$ T cell responses require $CD4^+$ T cell help (Shedlock and Shen, 2003).

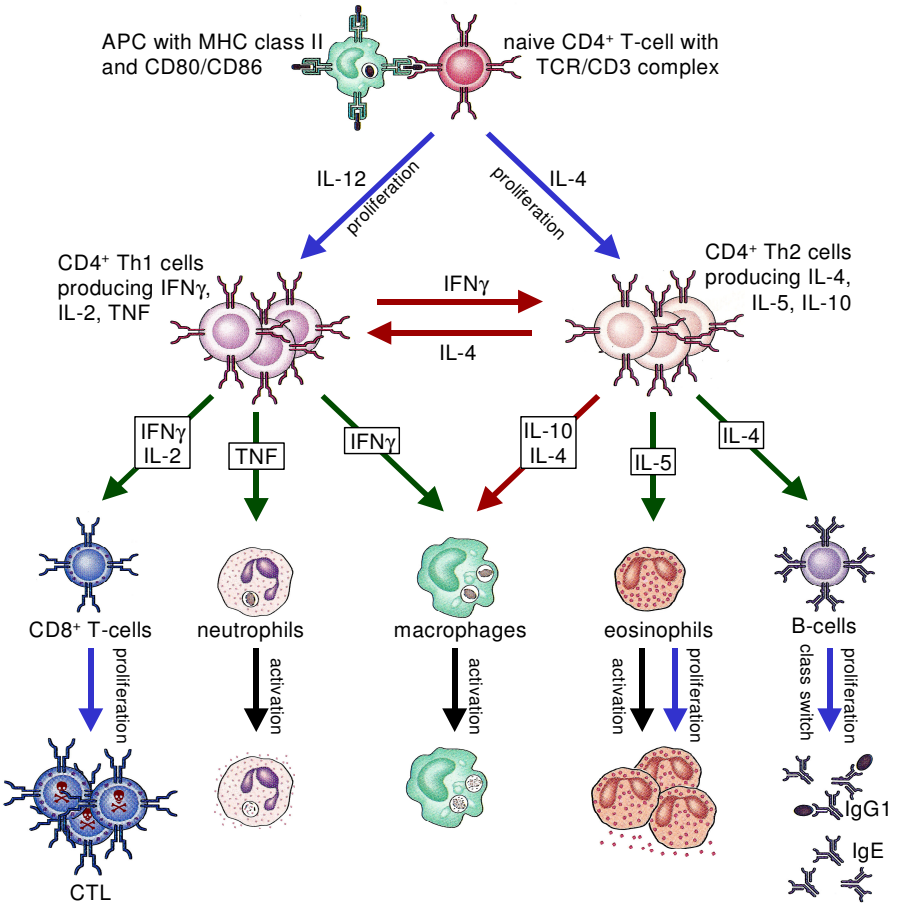


Figure **1**: Schematic representation of Th1 and Th2 cell development and selected effector functions. Naïve CD4+ T cells differentiate into Th1 or Th2 cells dependent on the presence of IL-12 or IL-4 at the time of APC contact. Cytokines secreted by the two CD4+ T cell types are indicated with their positive (green arrows) or negative (red arrows) effect on proliferation (blue arrows) or activation (black arrows) of other cells of the immune system. Parts of this figure were adapted from the book Cellular and Molecular Immunology by A.K. Abbas, A.H. Lichtman and J.S. Pober (Abbas et al., 2000)

$CD4^+CD25^+$ T (Treg) cells comprise an auto-reactive T cell subset that regulates and suppresses immune responses. Although the regulatory mechanism remains unclear, secretion or expression of either IL-10, TGF-β, CTLA-4 or a combination of these molecules appears to be involved (Mills, 2004). In addition to Treg cells, another important self-reactive T-cell sublineage was identified, Natural Killer T (NKT) cells.

NKT cells posses both regulatory and effector functions, are self-reactive and express a semi-invariant T-cell receptor (TCR) specific for conserved glycolipid ligands (Bendelac et al., 1997). In contrast to Treg cells, which have a dedicated suppressor function, NKT cells can facilitate autoimmune pathology as well as take part in protective mechanisms (Table 1).

Table **1**: Comparison of Treg cells and NKT cells

Feature	Treg cells	NKT cells
Distribution	Thymus, lymph node, spleen, circulation, sites of inflammation	Thymus, spleen, circulation, liver, bone marrow, sites of inflammation
Prevalence (spleen and thymus)	5 - 10 %	0.5 - 1 %
TCRs	Diverse	Invariant Vα, limitied Vβ
Co-receptors	Mostly CD4	CD4 or double neagative
Specificity	Diverse autologous peptides bound to MHC class II. Capable of recognizing non self peptides	Autologous and bacterial glycosphingolipids presented by CD1d
Effector functions	IL-10, TGFβ, CTLA-4, cytotoxicity? Other?	Diverse Th1 and Th2 cytokines, chemokines, FasL, CD40L
Mode of action	Suppression	Suppression or activation

1.5 Antigen presentation

Antigen processing and presentation to T cells with specific recognition of these antigens by the TCR of the T cells is essential for elicitation of a T cell response. Protein antigens can be processed and presented by two pathways. Cytoplasmic peptides are presented by MHC class I molecules and proteins from the endosomal pathway are presented via MHC class II molecules. Cytoplasmatic proteins, such as self-proteins or proteins derived from viruses and some intracellular bacteria are degraded to peptides in a proteolytic enzyme complex, the proteasome. The peptides are thereupon transported into the endoplasmatic reticulum (ER)-lumen in an ATP-dependent fashion by the transmembrane proteins TAP-1 and TAP-2 (Goldberg and Rock, 1992; Shepherd et al., 1993). Binding of these peptides by newly synthesized MHC class I molecules induces a conformational change within the MHC class I protein which enables the release of MHC class I from the chaperones calnexin and calreticulin and the binding of β2-microglobulin (β2m) (Degen et al., 1992). Complete MHC class I-β2m-peptide complexes are transported to the cell surface where they can be recognized by the TCR of $CD8^+$ T cells in a peptide specific manner (York and Rock, 1996) (Figure 2).

MHC class II molecules present peptide fragments derived from proteins taken up via phagocytosis and which are processed by the phagosome-endosome pathway (Ramachandra et al., 1999). Moreover, MHC class II molecules have access to peptides of some pathogens like mycobacteria which are taken up by macrophages but manage to survive and replicate inside the phagosome of the host cell (Ramachandra et al., 2001). During phagosomes maturation, phagosomes get acidified and thereby activate proteases such as cathepsins. These proteases cleave proteins to peptide fragments which are loaded onto MHC class II molecules. This process takes place in MHC class II positive vesicles (MIIC compartment) and is catalysed by HLA-DM (human) and H2-M (mice) (Watts, 1997). The transit of MHC class II proteins to the MIIC is mediated through binding of the MHC class II protein to the invariant chain (Li) which possesses a sorting sequence for the MIIC (Denzin and Cresswell, 1995). Li is cleaved in an acidified endosome, leaving a short peptide fragment, CLIP, still bound to MHC class II. Binding of LI and CLIP to MHC class II proteins prevents binding of peptides to MHC class II molecules in the ER. HLA-DM binds to MHC class II molecules, releasing CLIP and allowing binding of peptides to MHC class II molecules. Peptide-MHC class II complexes travel to the cell surface and are specifically recognized by $CD4^+$ T cells (Figure 2).

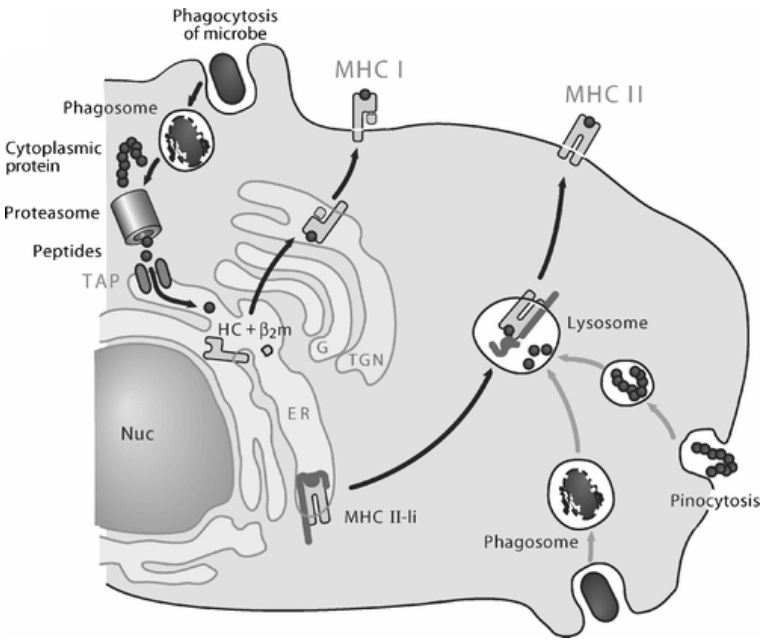


Figure **2**: Intracellular trafficking of MHC class I and II molecules. MHC class I and II intracellular trafficking. MHC class I and II molecules are assembled in the endoplasmic reticulum (ER). MHC class I molecules associate with β2-microglobulin (β2m), acquire peptides delivered from the cytosol and follow the secretory route to the plasma membrane. MHC class II molecules assemble into invariant chain (Ii) complexes in the ER that prevent peptide binding and direct their trafficking to endosomal compartments. In lysosomes, the Ii is cleaved, peptides are loaded and MHC class II molecules traffic to the cell surface. These pathways allow peptide sampling from the cytosol and endosomal compartments by MHC class I and class II, respectively. Nuc, nucleus; HC, heavy chain; G, Golgi; TGN, trans Golgi network; TAP, transporter associated with antigen processing. This picture is adapted from the publication: CD1: Antigen presentation and T cell function, Manfred Brigl and Michael Brenner, Annu. Rev. Immunol. 2004.

MHC class I and class II molecules only present peptide antigens to $CD8^+$ and $CD4^+$ T cells, respectively. Lipid antigens, either endogenous or bacterial lipids, are presented by the alternative antigen presenting molecules of the CD1 family.

1.6 CD1 molecules

During the recent years it became clear that not only peptides are presented to T cells, but that T cells also recognize lipids antigens. CD1 proteins bind lipids to form CD1-lipid antigen complexes displayed on the cell surface of APCs which contact T cell receptors and activate T cells. The five, non-polymorphic CD1 genes are encoded in close proximity on chromosome 1 in humans and chromosome 3 in mice and are not associated with the mammalian MHC gene locus (Calabi and Milstein, 1986). The CD1 family of antigen presenting molecules is composed of five members: CD1a, CD1b, CD1c, CD1d and CD1e (Ernst et al., 1998). They can be classified into two groups: CD1a, CD1b, CD1c and CD1e comprising group I CD1 molecules, found in humans but not in mice, and CD1d belonging to the group II CD1 molecules, present in humans and mice. Mice express two CD1d genes, CD1d1 and CD1d2, with the latter having no assigned function (Gozalbo-Lopez et al., 2004). CD1a - CD1d are involved in antigen presentation (Brigl and Brenner, 2004; Kaufmann and Schaible, 2005) whereas the CD1e gene is expressed as differentially spliced protein products but has no know function in antigen presentation. However, it could be involved in facilitating ligand binding to CD1 proteins (Angenieux et al., 2000). CD1 molecules are evolutionary conserved and CD1 genes, unlike MHC class I and II genes, show very limited polymorphism (Brossay and Kronenberg, 1999). CD1 polypeptides are expressed as heterodimers composed of

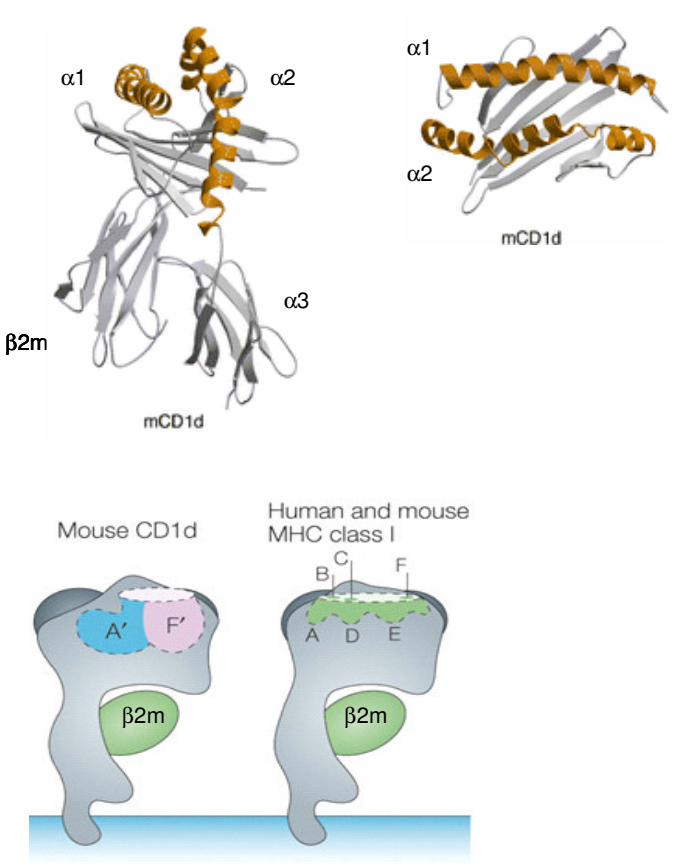


Figure **3**: CD1 structures. a) CD1 atomic structures. (left) Front and (right) top view of mouse CD1d (mCD1d) structure without ligand. α1-α3 domains and β2-microglobulin (β2M) are shown in light gray ribbons, α1 and α2 helices in brown. This picture was adapted from the publication: CD1: Antigen presentation and T cell function, Manfred Brigl and Michael Brenner, Annu. Rev. Immunol. 2004. b) CD1 pockets and portals. Two-dimensional schematic renderings of MHC class I and CD1 grooves, and their constituent pockets, were drawn based on the silhouettes of the crystal structures of mouse CD1d. The grooves of the CD1 proteins are deeper than those of the MHC class I molecules. Mouse CD1d contains two pockets for binding of the lipid chains, A' and F' This picture was adapted from the publication: Anatomy of CD1-lipid antigen complexes, Moody et al, Nature Reviews Immunology, 2005.

the CD1 heavy chain noncovalently paired with β2m (Figure 2). CD1 proteins are presented TAP-independently but association with β2m appears to be necessary for cell surface expression as shown by lack of surface localization in β2m-deficient cells (Sugita et al., 1997). The CD1 structure revealed a striking structural similarity to MHC class I in which the αchain folds into three domains, α1, α2, and α3 (Figure 2) (Zeng et al., 1997). However, the antigen binding groove of CD1 molecules is larger, deeper and more hydrophobic than the groove of MHC class I and II proteins. The CD1 isoforms have distinctly shaped antigen binding grooves and several recent crystal structures of antigen-CD1 complexes show how the lipid moieties of antigens are inserted into the deep hydrophobic antigen binding grooves. Thereby, the carbohydrates, peptides or other hydrophilic elements of the antigen are exposed to the surface of the CD1 molecule where they can directly contact the TCR (Figure 2) (Gadola et al., 2002; Zajonc et al., 2003).

The structural similarities to MHC proteins initially led to the idea that CD1 molecules could be involved in antigen presentation (Calabi and Milstein, 1986). The function of CD1 in T cell activation was discovered through studies of the direct recognition of CD1a and CD1c proteins in the absence of exogenous antigens, and the first known exogenous antigen identified as presented by CD1b was the large, unusually hydrophobic mycobacterial lipid mycolic acid (Porcelli et al., 1992; Porcelli et al., 1989). The most striking expression of CD1 molecules is found on DCs and professional APCs. CD1a, b and c are found on DCs whereas Langerhans cells express CD1a and CD1c but lack CD1b. CD1c is unique among group I CD1 proteins for its expression on B cells (Pena-Cruz et al., 2003; Porcelli, 1995; Smith et al., 1988). CD1d expression was detected for intestinal ephithelia, hepatocytes and hematopoietic cells like B and T cells, DCs and macrophages (Balk et al., 1994; Bleicher et al., 1990; Brossay et al., 1997)

1.6.1 CD1d

Despite the overall similarity in domain organization, striking differences between CD1d and MHC class I and II are found in the topography and molecular surfaces of the antigen-binding grooves (Zeng et al., 1997). For CD1d, the overall volume and surface area of the groove is larger than any MHC class I molecule, and the smaller pockets characteristic of MHC molecules are coalesced into two large pockets, designated A and F (Figure 3). The CD1d groove is closed at both ends but is accessible at the top of the molecule through a narrow opening. Few amino acid side chains that line the groove of CD1d are capable of hydrogen bonding. As a result, the likelihood of forming an extensive hydrogen bonding network at the ends of a peptide (as in class I) or along the sides of the longitudinal axis of the groove (as in class II) is not apparent in CD1d (Moody et al., 1997).

CD1 heavy chains are translocated into the ER where N-linked glycans are attached, interactions with ER chaperones occur, and association with β2m takes place (Sugita et al., 1997). CD1d associates in the ER with both calnexin and calreticulin and the thiol oxidoreductase ERp57. This process is coupled to disulfide bond formation in the CD1d heavy chain (Kang, 2002). It was suggested that lipid ligand binding to CD1d can occur in the ER (De Silva et al., 2002). CD1d contains a signal sequence in the cytoplasmatic tail which is responsible for localization to the late endosomal and MIIC compartments (Porcelli, 1995). Newly synthesized CD1b and CD1d molecules are rapidly delivered from the Golgi to the plasma membrane, presumably along the secretory pathway. CD1d molecules are internalized from the plasma membrane and traffic through early and late endosomes to lysosomes (Jayawardena-Wolf et al., 2001) (Figure 4).

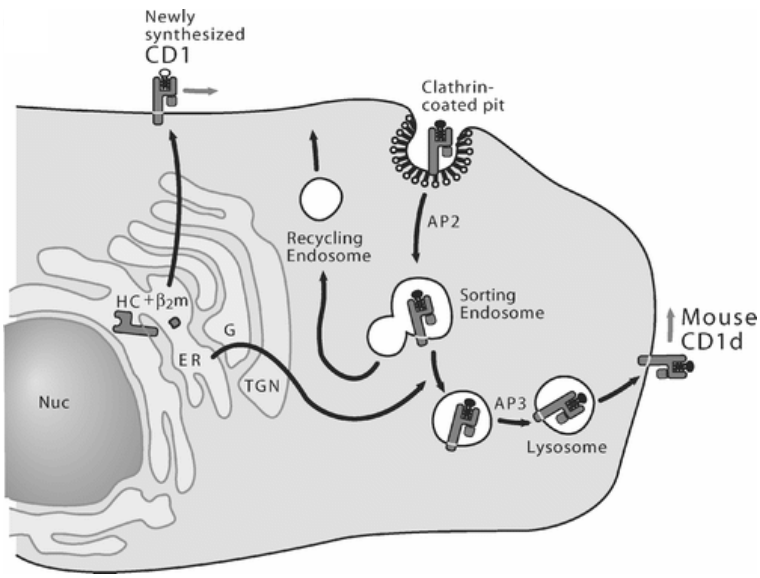


Figure **4**: Murine CD1d intracellular trafficking. Newly synthesized CD1d molecules may or may not assemble with β2m in the ER, acquire self-lipids, and traffic to the plasma membrane along the secretory route. A subset of CD1d molecules associate in MHC class II/Ii chain complexes and may be directed to endosomal compartments from the trans Golgi network (TGN). CD1d molecules are internalized from the plasma membrane and traffic through the early and late endosomal compartment and are delivered to lysosomes in an AP3-dependent manner. CD1d then acquires distinct self-lipid antigens in late endosomal/lysosomal compartments. This picture was adapted from the publication: CD1: Antigen presentation and T cell function, Manfred Brigl and Michael Brenner, Annu. Rev. Immunol. 2004.

1.7 Infection with mycobacteria

Pathogens can be categorized into two major groups based on their habitat. Intracellular pathogens reside and grow inside cells of the host whereas extracellular pathogens reside and grow in body fluids and extracellular spaces. Bacteria like streptococci, staphylococci as well as parasites and fungi such as *Candida albicans* belong to the group of extracellular pathogens. Viruses, some parasites (*Toxoplasma gondii, Leishmania major*) and a group of bacteria including *Listeria monocytogenes*, *Salmonella typhimurium* and pathogenic mycobacteria belong to the category of intracellular pathogens (Kaufmann and Hess, 1999).
Mycobacteria are aerob, gram$^+$, non-sporulating and immotile microorganisms. Many mycobacteria are widely found and e.g. can be isolated from soil. In contrast, pathogenic mycobacteria grow very slowly and need a complex nutrition. The pathogens causing

tuberculosis in humans (*Mycobacterium tuberculosis*) as well as bovine tuberculosis (*Mycobacterium bovis*) belong to this group. Infection with mycobacteria are problematic primarily because they mycobacteria are resistant to most common antibiotics and chemotherapeutic agents (Jarlier and Nikaido, 1994).

1.7.1 The mycobacterial cell wall

Mycobacteria are characterized by a stable, rigid and hydrophobic cell wall which protects against lysosomal enzymes, reactive oxygen and nitrogen metabolites and other defense mechanisms of the host. The cell wall is composed of a complex mixture of various compounds and is unique for mycobacteria (Brennan and Nikaido, 1995). The primary cell wall structure is composed of a plasma-membrane stabilized by a murein backbone. Attached to this is a arabinogalactan layer, esterified with mycolic acids. Free lipids and a capsule composed of phosphatidylinositol-mannosides (PIMs) are attached to the outer face of the cell wall. The plasma membrane is mainly composed of phosphatidylethanolamin, phosphatidylinositol and diphosphatidylglycerol (Cardiolipid, CL). Lipoarabinomannan (LAM) is a major constituent of the mycobacterial cell wall. LAM is composed of a PIM core, anchored to the plasma membrane and a polymer composed of arabinose residues to which mannose residues are attached.

1.7.2 Immune responses to mycobacterial infection

Mycobacteria have developed extensive mechanisms to avoid degradation by the microbicidal arsenal of macrophages and to interfere with lymphocyte activation. Mycobacteria mainly persist intracellularly in phagosomes of macrophages. Phagocytosis of bacteria usually leads to their degradation in the endosomal pathway. Endosomes mature, are acidified and fuse with lysosomes which contain a variety of decomposition enzymes like proteases and lipases as well as reactive oxygen and nitrogen metabolites (Kornfeld and Mellman, 1989). However, mycobacteria, although taken up into the phagosome, prevent their degradation by the host. They inhibit the acidification of the phagosome and the fusion of the phagosome with endosomes and lysosomes which disconnects them from the endosomal/lysosomal maturation and degradation pathway (Hasan et al., 1997; Sturgill-Koszycki et al., 1994). Moreover, they compete with the host for intracellular iron (Russell et al., 1996; Schaible et al., 1998). Components of the mycobacterial cell wall are responsible for mycobacterial survival within phagosomes of macrophages through the modulation of macrophage functions and lymphocyte stimulation (Chan et al., 1991a; Vergne and Daffe, 1998). Mycobacterial LAM inhibits T cell stimulation and Protein Kinase C activity, interferes with IFNγ mediated macrophage activation and neutralizes oxygen radicals (Kaplan et al.,

1987). Additionally, inhibition of antigen-specific monocyte activation can be inhibited by PIMs.

However, there are several ways the immune system competes with mycobacterial infection. IFNγ activated macrophages exhibit a range of anti-mycobacterial devises. Activated macrophages downregulate the amount of transferrin-receptor and ferritin leading to decreased availability of iron within the phagosome (Schaible and Kaufmann, 2004). Furthermore, INFγ induces the production of reactive oxygen and nitrogen metabolites by the macrophage empowering them to attack and destroy intracellular bacteria (Fazal, 1997). Mycobacterial protein antigens are presented by MHC class II molecules to $CD4^+$ T cells. These T lymphocytes are essential for establishing an immune response to mycobacteria, since mice lacking $CD4^+$ T cells are highly susceptible to mycobacterial infection (Caruso et al., 1999). The main function of these T cells in defence against mycobacteria is the production of IFNγ. Despite $CD4^+$ T cells, other lymphocytes are important for anti-mycobacterial immune responses. Cross presentation mediates activation of $CD8^+$ T cells which lyse infected target cells with the residing mycobacteria via perforin / granzme B and also secrete IFNγ (Flynn and Chan, 2001; Schaible et al., 2003). Antibody production against mycobacterial lipids and proteins was detected in human tuberculosis patients. Although antibodies against mycobacterial LAM, PIM, trehalose dimycolate and cardiolipin could be detected, these do not play a role in immune defence against mycobacteria (Fournie et al., 1991; Julian et al., 1997; Pan et al., 1999; Santiago et al., 1989). Furthermore, lipids shed by mycobacteria during infection (Fischer et al., 2001) can be presented by CD1 molecules and are recognized by lipid-antigen specific T cells. These lipid specific T cells could have an important role in immune responses against bacterial pathogens (Moody et al., 2000)

1.8 CD1 lipid antigens and CD1 reactive T cells

CD1 molecules, except CD1a contain a signalling sequence that direct their trafficking to late endosomes and lysosomes (Porcelli, 1995). Mycobacterial lipids have access to these compartments as well. It was shown that lipoarabinomannan (LAM) is taken up via the mannose receptor and could be detected in late endosomes and MIIC compartments together with CD1b. This implies that CD1 molecules could meet different (mycobacterial) lipid antigens in specialized compartments, bind them and present them on the cell surface to T cells (Prigozy et al., 1997). This is supported by the presence of a wide range of degradative enzymes, such as glycosidases and lipases in the acidified endosomal/lysosomal vesicles. These enzymes could be involved in the processing of lipid antigens, derived from the mycobacterial phagosome (Reimann and Kaufmann, 1997).

The identification of the first antigen presented by CD1 molecules to T cells demonstrated that CD1 presented antigens are lipids (Beckman et al., 1994). So far, it has been shown that both hydrophobic peptides and lipids/glycolipids can be presented by CD1 molecules (Porcelli and Modlin, 1999). The foreign antigens presented by CD1 molecules that have been characterized include a range of diverse lipids found in the cell walls of mycobacteria. Mycolic acid, glucose-monomycolate (GMM) and lipoarabinomannan (LAM) are presented by CD1b molecules (Beckman et al., 1994; Moody et al., 1997; Sieling et al., 1995).

Mycobacterial mannosyl-β1-phosphoisoprenoid, a glycophospholipid with only a single short lipid tail, is recognized by a CD1c-restricted T cell line (Moody et al., 2000). CD1a-restricted T cells recognize a lipopeptide (didehydroxymycobactin) from *Mycobacterium tuberculosis*, defining a new biochemical class of antigens for CD1-restricted T cells (Moody et al., 2004). T cells that are group I CD1 restricted display a $CD4^-CD8^-$ double negative (DN) or $CD8\alpha/\beta^+$ or $CD4^+$ phenotype. DN T cells predominantly express an α/β-TCR but can also carray a γ/δ-TCR (Shamshiev et al., 2000; Spada et al., 2000). The common structural feature of all lipid antigens presented by CD1a, b and c is a hydrophobic moiety composed of 2 acyl chains which bind to the 2 hydrophobic pockets of the CD1 molecule, and a polar head group displayed on the surface of the CD1 molecule and recognized by the TCR. Removal of acyl chains leads to loss of CD1 binding and removal or modification of the polar head groups diminishes T cell recognition and activation (Moody et al., 1997).

Microbial lipids or lipopeptide antigens presented by CD1 have been confirmed only for CD1a, b, and c. There have been conflicting reports whether murine CD1d-restricted T cell (NKT cells) recognize glycosylphosphatidylinositol (GPI)-anchored glycoproteins from *Plasmodium* or *Trypanosoma* spp. in vitro or affect the IgG response to GPI-linked proteins in vivo (Duthie et al., 2002; Procopio et al., 2002; Romero et al., 2001; Schofield et al., 1999). Mycobacterial phosphatidyinositol-dimannosid (PIM_2) was shown to elicit a NKT cell response. However, this response was CD1d independent (Gilleron et al., 2001). Until recently, the only CD1d-presented antigen that elicits a NKT cell response was α-Galactosyl-ceramide (αGalCer) (Kawano et al., 1997). It strongly induces actvation of NKT cells and rapid production of cytokines. However, this sphingolipids is derived from the marine sponge *Agelas mauritianus* and is of unclear physiological relevance.

1.9 NKT cells

Unlike classical T cells reactive to peptides presented by MHC molecules, NKT cells are reactive to lipid antigens presented by CD1d. The majority of NKT cells can be either $CD4^+$ or $CD4^-CD8^-$ double negative and express a very limited range of TCR variable region genes. In mice, most NKT cells express an invariant Vα14Jα18 TCR with predominant coexpression of Vβ8, Vβ2 and Vβ7. Human NKT cells express a Vα24Jα18 rearranged TCR α chain typically coexpressed with Vβ11. CD1d and NKT cells are evolutionary highly conserved with mouse NKT cells recognizing human CD1d and vice versa (Bendelac et al., 1997; Bendelac et al., 1995; Brossay and Kronenberg, 1999; Dellabona et al., 1994). Most NKT cells express the NK cell marker NK1.1 (NKR-P1C). However, since NK1.1 expression is variable this description is not sufficient. Many conventional T cells upregulate NK receptors upon stimulation while many CD1d-restricted NKT cells do not express them (Godfrey et al., 2004). The most accurate description of the majority of NKT cells is expression of an invariant TCR and reactivity to αGalCer as can be detected with αGalCer-CD1d tetramers (Gumperz et al., 2002). The relative frequency of these cells varies in a tissue-specific fashion, the significance of which is not known (Hammond et al., 1999). Murine NKT cells are found at the highest frequency in liver (10 - 40% of liver lymphocytes) and with lower frequencies (≤1%) in thymus, bone marrow, spleen, lymph node, and blood (Emoto et al., 1999a). The distribution of human NKT cells in different tissues is not well defined but NKT cells are clearly less frequent (≤1%) in human liver compared with the NKT cell numbers in the liver of mice (Exley et al., 2002; Kenna et al., 2003).

NKT cells were initially characterized by their autoreactivity to CD1 molecules (Bendelac et al., 1995). Their surface phenotype resembles activated memory T cells even in the absence of exogenous stimulation indicating that self lipids presented by CD1d are sufficient for a certain degree of activation (Park et al., 2000). NKT cell activation by TCR ligation leads to extensive production of various cytokines within 1 - 2 hours after stimulation (Godfrey et al., 2000). NKT cells release substantial amounts of Th1 cytokines like IFNγ and TNF but also Th2 cytokines like IL-4, IL-10 and IL-13 (Chen and Paul, 1997; Mendiratta et al., 1997; Smyth and Godfrey, 2000) (Figure 5). They appear to store pre-formed mRNA for some of these cytokines even in the absence of activation by exogenous antigens (Matsuda et al., 2003; Stetson et al., 2003). The simultaneous expression of Th1 and Th2 cytokines by the same cell is a unique hallmark of NKT cells. However, the mechanism which determines the cytokine production profile of NKT cells is unknown. αGalCer induces both IFNγ and IL-4 secretion whereas OCH, an analogue of αGalCer with a truncated sphingosine chain, induces predominantly IL-4 (Miyamoto et al., 2001). Due to the production of Th1 and Th2 cytokines, NKT cells have been implicated both as pro-inflammatory lymphocytes that

enhance cell-mediated immunity and immunosuppressive cells, respectively (Smyth and Godfrey, 2000). Apart from cytokine production and immune regulation, NKT cells express effector molecules like CD40L and FasL (Fujii et al., 2004; Lisbonne et al., 2004).

NKT cells are involved in various immune responses. They play a role in suppression of tissue destruction, autoimmunity, antitumour responses, host defence, allergy and inflammation (Akbari et al., 2003; Chan et al., 2003; Cui et al., 1997; Gumperz and Brenner, 2001; Sakai et al., 1999; Seino et al., 2001; Sonoda et al., 2001; Terabe and Berzofsky, 2004; Van Kaer, 2004) (Figure 5). NKT cells participate in protection of mice from a variety of bacterial, viral, and protozoan parasites, although some of these results are controversial (Behar et al., 1999; Emoto et al., 1999a; Emoto et al., 1997; Flesch et al., 1997; Godfrey et al., 2000; Pied et al., 2000). However, IFNγ secretion was shown to be important (Kronenberg and Gapin, 2002). This does not mean that NKT cells act solely as effectors, since the majority of IFNγ secretion could be due to NK cells, stimulated as a result of NKT cell activation (Carnaud et al., 1999; Hayakawa et al., 2001). Furthermore, it is unresolved to which extent NKT cells respond directly to microbial glycolipids or whether activation of NKT cells by microbes is indirect. So far, knowledge is limited about the mechanism underlying NKT cell activation. It was demonstrated that NKT cells can be activated and recruited to sites of infection in a CD1d-independent manner (Gilleron et al., 2001).

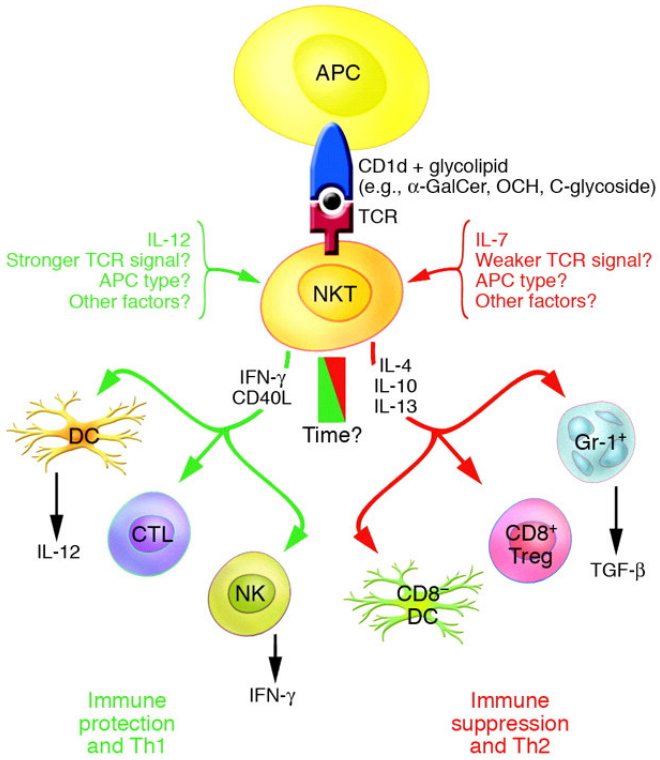


Figure **5**: Schematic outline of how NKT cells influence the immune response in either a Th1 or Th2 direction. The green part shows factors supporting the Th1 responsiveness by NKT cells, while the red part shows factors supporting Th2 responsiveness by these cells. The environment in which NKT cells are found, including the type of cytokines and APCs and the strength of antigen-mediated TCR stimulation they receive, determines the cytokine output. Upregulation of cell-surface CD40L by NKT cells stimulates IL-12 release by DCs, which cooperate with NKT cell-derived IFNγ to stimulate Th1 responses. IL-7 has been reported to enhance Th2 cytokine production by NKT cells, while cell-surface molecules that mediate immune suppression or Th2 deviation by these cells have not been identified. Time after stimulation may also be an important factor, as NKT cells also tend to produce Th1- and Th2-like cytokines in the short term (1-3 hours) following stimulation but may then switch to a Th1 output, which can last for several days. This picture was adapted from the publication: Going both ways: immune regulation via CD1d-dependent NKT cells, Dale I. Godfrey and Mitchell Kronenberg, JCI, 2004

Furthermore it was shown that proinflammatory cytokines secreted by APCs after exposure to microbial products augmented the basal weak responses of CD1d-restricted T cells to unknown self antigens to yield potent effector functions. Recognition of microbial products and activation of APCs was mediated by TLRs expressed on the APC surface (Brigl et al., 2003) (Figure 6). This mechanism could account for rapid, indirect activation of CD1d-restricted T cells in a variety of microbial infections. However, it was demonstrated that NKT cells directly and specifically recognize antigens presented by CD1d. This mechanism depends on antigen-uptake, -processing and -presentation by APCs and is antigen-specific and sensible to modifications of the antigen structure (Kawano et al., 1997) (Figure 6).

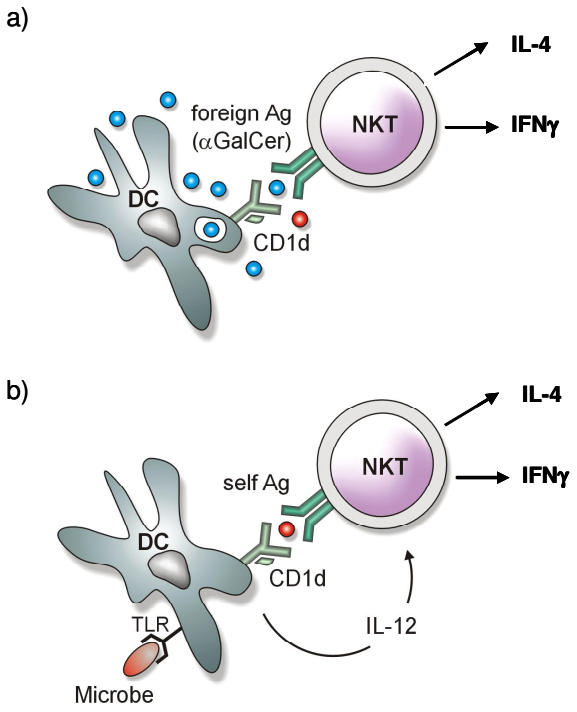


Figure **6**: Models of NKT cell activation. a) Foreign lipid antigens are presented via CD1d on the cell surface of professional APCs and are sprecifically recognized by the NKT cell TCR leading to activation of NKT cells. b) Professional APCs presented endogenous NKT cell ligands via CD1d and "pre-activate" NKT cells by providing the first signal for activation. TLRs expressed on APCs mediate recognition of microbes by the APCs thus leading to activation and production of IL-12 by APCs. IL-12 in turn activates NKT cells, thus providing the second stimulation signal. Picture is courtesy of Stefan H.E. Kaufmann

It remains to be elucidated how NKT cell activation is regulated and which mechanisms determine the type of NKT cell response and its influence on the systemic immune system. Moreover, an analysis of the genetic décor of NKT cells could provide a mean for a global characterisation and an understanding of NKT cell functions in diverse immune responses.

2 Aims of this thesis

NKT cells are a unique T lymphocyte lineage that has been implicated in the regulation of immune responses associated with a broad range of diseases, including autoimmunity, infectious diseases, and cancer. In stark contrast to both conventional T lymphocytes and other types of regulatory T cells (Treg), NKT cells are reactive to the nonclassical antigen-presenting molecule CD1d, and they recognize glycolipid antigens rather than peptides. Moreover, they can either up- or downregulate immune responses by controlling the secretion of Th1, Th2, or immune regulatory cytokines.

Although there are numerous publications describing NKT cells in infection and disease, little is known about the properties of NKT cells and the mechanisms of NKT cell activation on a molecular level. Moreover, there is little knowledge about bacterial and endogenous ligands for NKT cells. Until recently, αGalCer, a sphingolipid from a marine sponge, was the only described agonist for NKT cells. A preceding study from this group identified Phosphatidylinosotol-mannoside (PIM) as lipid antigen, presented by CD1d and recognized by NKT cells (Fischer et al., 2004). In 2005 a publication by Zhou et al demonstrated that NKT cells recognize mammalian iGb3 and characterized this lipid as the endogenous NKT cell antigen (Zhou et al., 2004).

The aims of this thesis are:

- characterization of NKT cell properties and functions by analysis of their gene expression profile;
- identification, characterization and analysis of endogenous NKT cell antigens;
- analysis of NKT cell activation by endogenous ligands during lipid storage disease;
- characterization of PIM-reactive NKT cells;
- identification of mycobacterial lipids stimulating NKT cells via direct or indirect activation.

In summary, this thesis aims to define NKT cell properties by examination of their gene expression profile and to identify and characterize NKT cell antigens. Transcriptome analysis of naïve and activated NKT cells should allow to coalesce the diversity of NKT cell functions to their gene expression décor. Identification of new lipid antigens as well as characterization of PIM reactive and iGb3 reactive NKT cells was performed to get a detailed view of NKT cells during infection and disease.

3 Material

3.1 Cells, Cell lines

A20	Murine B-Cell lymphoma line (ATCC)
A20-mCD1d	A20 Cells transfected with DNA for the full-length murine CD1d molecule (kindly provided by Dr. Mitchell Kronenberg, La Jolla Institute of Allergy und Immunology, CA, USA)
NKT NAD II	Human NKT cell line
B-EBV Bois	Human B-Ebstein-Bar-Virus transfected cell lines

3.2 Cell culture medium

Dulbecco´s Modified Eagle´s Medium (DMEM), RPMI 1640 medium, human serum, fetal bovine serum, penicillin/streptomycin solution, L-glutamine, HEPES, sodium pyruvate and non-essential amino acid solution were obtained from *Biochrom*.

Standard DMEM and RPMI medium:	10%	FCS
	2mM	L-glutamine
	1mM	sodium pyruvate
	50 µM	Mercaptoethanol
	10U/ml	penicillin and streptomycin
	10 mM	HEPES
	For studies with human cells, cell culture medium was supplemented with 8% human serum instead of FCS	
Macrophage medium	Complete DMEM or RPMI with 10-30% L-cell supernatant from L929 cells	

3.3 Bacteria

Mycobacterium bovis bacille Calmette Guérin (*M. bovis* BCG)	Statens Institut, Kopenhagen, Dänemark

3.4 Bacteria Culture medium

Luria Bertani (LB) medium:

1% (w/v) Bacto-Tryptone, 0.5% (w/v) yeast extract and 85.5 mM NaCl were dissolved in autoclaved water, the pH was adjusted to 7.5 with NaOH and the medium was autoclaved. Were indicated the appropriate antibiotics were added. Ampicillin was used at 50-100 mg/l. For plates 1.5 % (w/v) agar was added prior to autoclaving.

Middlebrook medium:
4,7g Middlebrook 7H9 Broth, 2ml Bacto glycerol and 0,5g Tween 80 were dissolved in 900ml ddH_2O and autoclaved. Subsequently 100ml Bacto-ADC enrichment was added under sterile conditions. Materials were purchased from Difco, USA.

3.5 Mice

Mice used in the experiments were on the genetic background of C57BL/6 (H-2^b) mice and between 6-10 weeks old.
Apart from wildtype mice, experiments were performed using knock-out mice with a deficiency for the CD1d gene. Moreover, mice with a transgene expression of the Vα14-Jα281 TCR were used.
Transgenic animals, C57BL/6 mice and CD1d $^{-/-}$ mice were bred in our facility at the Federal Institute for Health Protection of Consumers and Veterinary Medicine (BGVV) in Berlin. Mice were kept under specific pathogen-free (SPF) conditions in filter bonnet cages with food and water ad libidum. The experiments were conducted according to the German animal protection law.

3.6 Human samples

Blood samples from patients with Fabry disease were obtained from the University Children's Hospital of the University of Mainz in collaboration with Professor Dr. M. Beck. Blood samples from healthy donors were obtained from people working at the MPI for Infection Biology.

3.7 Antibodies

Monoclonal antibodies (mAb) used are listed in the table below**.** Monoclonal antibodies (mAb) marked with an asterisks (*) were purified from hybridoma supernatants by protein-G sepharose. The mAb used for flow cytometry were conjugated with the fluorescent dyes FITC, PE, APC, Cy-5 or PE-Cy-7. ATCC = American Type Culture Collection

Anti-mouse antibodies

Specificity	Clone	Application	Source
ATAC / Lymphotactin	IGH6	FACS	Dr. R. Kroczek
B220 / CD45	RA3-6B2	FACS	Pharmingen
CD3	145-2C11	FACS	Pharmingen
CD4	YTS191.1	FACS	ATCC*
CD8α	YTS169	FACS	ATCC*
CD11a	2D7	FACS	Pharmingen
CD11c	HL3 N418	FACS	Pharmingen ATCC

Specificity	Clone	Application	Source
CD16/CD32 (anti-FcR)	24G2	FACS	ATCC*
CD25	PC61	FACS	Pharmingen
CXCR4	2B11	FACS	ATCC*
F4/80	F4/80	FACS	ATCC*
FasL	MFL-3	FACS	ATCC*
IgG1		FACS	ATCC*
IL-4	11B11	ELISA	ATCC*
IL-4	BVD6-24G2	ELISA	ATCC*
IL-4	11B11	FACS	Pharmingen
IFNγ	XMG1.2	ELISA	ATCC*
IFNγ	R4-6A2	ELISA	ATCC*
IFNγ	XMG1.2	FACS	ATCC*
Mac-1	MI/70	FACS	Pharmingen
NK1.1	PK136	FACS	Pharmingen
anti-CD1d	20H1, 1B1	FACS	Dr. M. Kronenberg
Thy1.2	53-2.1	FACS	Pharmingen
TLR 2		FACS	Dr. Egil Lien
TLR 4	MTS510	FACS	eBioscience
TNFα	XT22	FACS	ATCC*

Anti-human antibodies

Specificity	Clone	Application	Source
CD3	UCHT1	FACS	Pharmingen
CD161/NK1.1	DX12	FACS	Pharmingen
CD1b	M-T101	FACS	Pharmingen
Invariant NKT	6B11	FACS	Pharmingen
FcR block		FACS	Miltenyi
CD1d	CD1d42	FACS	Pharmingen

Additional materials for FACS and ELISA

Specificity	Application	Source
Anti-rat PE	FACS	Pharmingen
DAPI	FACS	Pharmingen
PI	FACS	Pharmingen
Streptavidin - PE	FACS	Pharmingen
Streptavidin - PE-Cy7	FACS	Pharmingen
Streptavidin - POD	ELISA	Dianova

3.8 Primers

The table below shows the sequences of the synthetic oligonucleotides used for semi-quantitative RT-PCR. All primers were obtained from *Thermo Electron* and diluted to 100 µM stock solutions. The working solutions were prepared from stock solutions according to the requirements. RT-PCR primer sequences were chosen using the Primer express 2.0 software from *Applied Biosystems*.

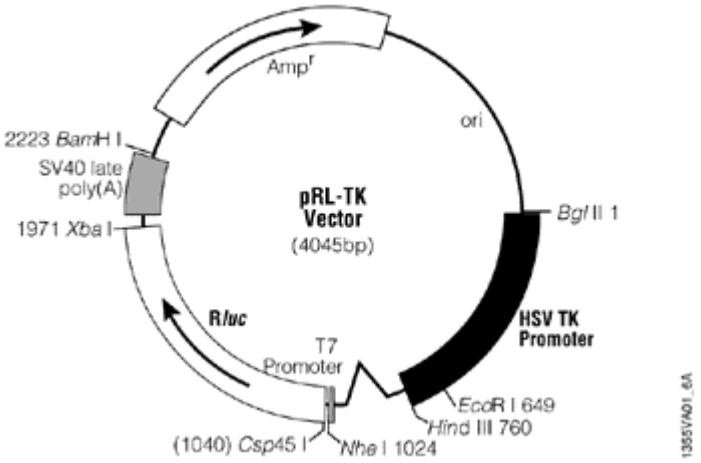
Primer-name	**5'-3' sequence**	**Application**
mIFNγ Fwd	CTGCTGATGGGAGGAGATGTCT	RT-PCR
mIFNγ Rev	TGCTGTCTGGCCTGCTGTTA	RT-PCR
mIL-13 Fwd	TTCCCTGACCAACATCTCCAA	RT-PCR
mIL-13 Rev	GGGCCTTGCGGTTACAGA	RT-PCR
mIL-17 Fwd	CCCTTGGCGCAAAAGTGA	RT-PCR
mIL-17 Rev	CGTGGAACGGTTGAGGTAGTC	RT-PCR
mBLK Fwd	TGAGACTCCCAGCATGAAGGA	RT-PCR
mBLK Rev	GCCTTCCACGCACTCCAT	RT-PCR
mCCR3 Fwd	GCACCACCTCAGTGCTTATGAC	RT-PCR
mCCR3 Rev	TGGGAACGTGTTGTTGATGCT	RT-PCR
mIL-17B R Fwd	GCGACTATAATGCCCTGAGTGTCT	RT-PCR
mIL-17B R Rev	GAAAGCTGTGGCGTCCTTCA	RT-PCR
mKLF2 Fwd	CCGCCACTACCGAAAGCA	RT-PCR
mKLF2 Rev	TCGCACAAGTGGCACTGAA	RT-PCR
mL-sel Fwd	TCAAGTCCTCCCGTGAAGATTT	RT-PCR
mL-sel Rev	CGTTGAGCAAACACTGCATCTC	RT-PCR
mATAC Fwd	TGGATGGCAGGGCCAGTA	RT-PCR
mATAC Rev	TGGGCTCCTGTGGGAACA	RT-PCR
mFasL Fwd	GCAGCAGCCCATGAATTACC	RT-PCR
mFasL Rev	AGATGAAGTGGCACTGCTGTCTAC	RT-PCR
mCCL5 Fwd	CCAATCTTGCAGTCGTGTTTGT	RT-PCR
mCCL5 Rev	TCTCTGGGTTGGCACACACTT	RT-PCR
mGAPDH Fwd	CTCGTCCCGTAGACAAAATGG	RT-PCR
mGAPDH Rev	TGACCAGGCGCCCAATA	RT-PCR

3.9 Plasmids

pgD-hTLR2 — Full length hTLR2 with NH2-terminal gD tag (Yang et al., 1998). The wild type signal sequence for hTLR2 was replaced by the signal sequence and an epitope tag for herpes simplex virus type 1 glycoprotein D. (Mark, JBC, 1994).

p pGL3-ELAM.tk: Firefly luciferase vector (Yang et al., 1998)) (*Promega*)

pRLTK (*Promega*):



3.10 Chemicals

Addresses and contact information for suppliers are attached in section 13. Standard laboratory chemicals used to prepare buffers were purchased from *Sigma, Merck* or *Roth* in per analysis quality. Radioactive compounds were obtained from *Amersham* (England) or *NEN* (USA).

αGalCer	*Kirin Brewery Co. LTD*
CD40L	*Alexis Biochemicals*
FuGENE	*Promega*
Gb3	*Matreya Inc.*
iGb3, P15, PW16, PW17, PW18	*Dr. P. G. Wang, (Department of Chemistry, Wayne State University, Detroit, USA)*
iGb3	*Dr. A. Bendelac (Committee on Immunology, University of Chicago, Chicago, USA)*
iGb3	*Alexis Biochemicals*
Jirimycin	*Sigma*
Lactosylceramide	*Sigma*
Magnetic cell sorting beads	*Miltenyi Biotec*
Recombinant αGalA	*Transkaryotic Therapies*
Recombinant murine and human cytokines	*Strathmann Biotec AG*
Cyanine 3-CTP, Cyanine 5-CTP	*NEB Life Science Products*

3.11 Buffers and solutions

Solutions were made up in H_2O prepared with a *Millipore* water purifier, unless stated otherwise. Where indicated, solutions were sterilized by autoclaving for 25 min at 121°C, or filter-sterilized through a 0.2 µm membrane.

Antibody Solution	0.1 % BSA 0.1% Tween 20 0.1% sodium azide in 1x PBS	Silver Stain developer	3% sodium carbonate 0.5% formaldehyde in ddH_2O
ELISA coating buffer	PBS or 100 mM Na carbonate / hydrogencarbonate titrated to pH to 9.6	Silver stain fixing solution	10% Essigsäure 30% Methanol in ddH_2O
ELISA wash buffer	0.1% BSA 0.05 % Tween 20 in PBS	Fixing buffer	50 ml Ethanol and 10 ml Essigsäure with 40 ml ddH_2O
ELISA Blocking buffer	PBS/0.1% BSA	Separating gel buffer (4x)	90.75g Tris Base 20ml 10% SDS volume to 460mls pH to 8.8 with HCl Q.S. to 500ml
ELISA Detection buffer	PBS/0.1% BSA	Stacking gel buffer (4x)	12.12g Tris Base 8ml 10% SDS bring volume to 180mls pH to 6.8 with HCl Q.S. to 200ml
ELISA substrate buffer	Citric Acid Buffer (0,1M; pH5) with 0.1% H_2O_2	Sample buffer (3 x)	187.5 mM Tris-HCl, pH 6.8 6% SDS 0.9 mM EDTA 30% glycerol 0.03% bromophenol blue 7.5% b-mercaptoethanol
ELISA stop solution	20% H_2SO_4	Running buffer	25 mM Tris-HCl 0.1% SDS 192 mM glycine, pH to 8.3
Phosphate buffered saline (PBS):	8g NaCl 0.2g KCl 0.2g KH_2PO_4 1.3g Na_2HPO_4 ad 1000 ml	Acrylamide	3.9 g Bisacrylamide 146.1 g Acrylamide ad 500 ml ddH_2O
Erythrocyte lysis buffer	8.3 g NH_4Cl 20.6 g Tris ad 1000ml ddH_2O adjust to pH 7.65	α-Galactosidase A assay substrate buffer	50 mM Citrate-Phosphate-buffer, pH: 4.5
Cell lysis buffer	TE buffer (pH 6.5) Protease Inhibitors: TLCK, E64, Pepstatin, Leupeptin ((1/1000) 8.55 % Sucrose	α-Galactosidase A assay stop buffer	12.6 g Glycin 18 g Na_2CO_3 ad 1000ml ddH_2O adjust to pH 10

3.12 Enzymes

T4 DNA ligase reverse transcriptase superscript II DNAse I RNAse H	*Gibco*
Pfu DNA polymerase DNA polymerase I, large fragment (Klenow)	*Stratagene*
Taq DNA polymerase	*Gene Craft*
2x SYBR-Green PCR master mix	*Applied Biosystems*

3.13 Equipment

FACS-Calibur, FACS-Canto, FACS-Diva	*Becton Dickinson*
Electrophoresis chambers	*Bio-Rad,* *Couldy Small II (Hoefer, USA)*
Electrophoresis Power Supply	*Bio-Rad*
Centrifuges	*Megafuge 2.0 R (Heraeus)* *Ultracentrifuge (Beckmann)*
ELISA-Reader SpectraMax 250	*Molecular Devices*
Microscopes	*Zeiss*
Lyophylizer Lyovac GT2	*Amsco/Finn-Aqua*
2100 bioanalyzer system DNA microarray laser scanner	*Agilent Technologies*
PCR thermocycler ABI DNA-Sequencer A377 ABI Prism 7000	*Applied Biosystems*
Scales	*BP 210 S (Sartorius)* *BP 2100 S (Sartorius)*
Sonicator / Ultrasonic Desintegrator	*Branson Sonifier W-250 (G. Heinemann)*
CO_2 Incubator	*Autoflow Incubator CO_2 4500 E (NUAIRE)*
Sterile Workbench	*Sicherheitswerkbänke HS 12 (Heraeus)*
Heating Block	
MALDI - Masspectrometer	*Voyager Elite (Perseptive Biosystems)*
pH - Meter 761 calimatic	*Knick*
Electronic pipetter	*Pipetboy acu (Integra Biosciences)*
Bacterial counting chamber	*Hausser Scientific Company*
Cell counting chamber	*Neubauer improved (Brand)*

Rotational Evaporator	*RotiVap (Büchi)*
Rocking Plate	*Biometra*
Vortex	*IKA-Labortechnik*
Stir- and Heatingplate	*IKA-Labortechnik*
Topcount NXT microplate scintillation counter	*PerkinElmer*
Filtermate 196 harvester	*PerkinElmer*
Luminometer monolight 3036 plate reader	*BD Bioscience*

3.14 Other material

Dialysis filter, Type VS, 0.0025 µm	*Millipore Corporation*
Sterile filters and membranes	*Schleicher & Schüll*
Flasks, plates and sterile vials	*Nunc*
TRIzol ® Reagent	*Gibco*
TLC plates (high resolution)	*Merck*
Chromatography equipment	*Pharmacia*
C18 Sep Pak chromatography columns	*Waters*
Chromatography support / media	*Pharmacia*
Unifilter microplates and seals	*PerkinElmer*
Microscint LSC cocktail	*PerkinElmer*
Biocoll, Percoll solution	*Biochrom*

3.15 Kits

Dual Luciferase reporter assay system	*Promega*
Human IFN-γ and IL-4 Cytoset ELISA Kit	*Biosource*
RNeasy mRNA extraction Kit	*Qiagen*

3.16 Software

Tables, calculations, statistics	GraphPad Prism 3.0 (*GraphPad Software*)
	Excel (*Microsoft*)
Graphics	PowerPoint (*Microsoft*)
	Photoshop (*Adobe Systems*)
Flow cytometric analysis	Cell Quest 3.0 (*BD*)
	FCS Express 2.0 (*De Novo Software*).
DNA primer design	Primer express 2.0 (*Applied Biosystems*)

Text	Word (*Microsoft*)
Microarray analysis	Resolver (*Rosetta Inpharmatics*)
ELISA Analysis	Softmax Pro, Spf 3.0 (*Molecular Devices*)
Mass-spectrometry analysis	Perseptive Grams/386 (Galactic Industries Corp.)

3.17 Web resources

DNA sequence identification:

http://www.ncbi.nlm.nih.gov/BLAST/

DNA sequence comparison:

http://www.ncbi.nlm.nih.gov/gorf/bl2.htlm

Oligonucleotide-primer design:

http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi

4 Methods

4.1 Immunological methods

4.1.1 Cell culture

Cell lines and primary cells were cultured under sterile conditions at 37°C, 95% humidity and 5 - 7% CO_2. At confluency, cells were passaged and diluted.
Adherent cells were detached by treating them with a Trypsin / EDTA solution for 2min (or until they start to detach) at 37°C and then carefully washed off and resuspended in complete medium. Semi-adherent cells were incubated with cold PBS for 10 min at 4°C and subsequently carefully detached using a cell scraper. A20 Cells were detached using a cell scraper.

To determine cell numbers and viability, an aliquot of the respective cell suspension was mixed with a Trypan blue solution (0.16%) and cell numbers were determined using a Neubauer cell counting chamber under the microscope. Cell vitality could be determined since living cells, in contrast to dead cells, actively export Trypan blue and do not stain blue.

4.1.2 Bacterial cell culture

M. bovis BCG was kept under sterile S2 conditions at 37°C in a shaking Middlebrook-media liquid culture. At a density of $2x10^8$ bacteria per ml, bacteria were passaged in a 1:20 dilution into fresh medium.

To determine the cell number, an aliquot of the liquid culture was drawn up and down a syringe with an 0.4 mm ∅ needle to homogenize the sample, transferred to a Petroff-Hausser bacterial counting chamber and counted under a microscope.

4.1.3 Infection of cells with *M. bovis* BCG

The number of bacteria and target cells were determined. Infections were done at a ratio of 10:1 (bacteria:target cells). The needed amount of bacteria was centrifuged at 10,000 x g and the supernatant was discarded. The bacterial cell pellet was resuspended in complete medium and washed once. Subsequently the bacterial cell pellet was homogenized by repeated transfer through a syringe with a 0.4 mm ∅ needle. After this, bacteria were added to the target cells.

4.1.4 Infection of mice with *M. bovis* BCG

C57BL/6 mice were infected i.v. with $2x10^6$ *M. bovis* BCG. Bacteria were grown in Middlebrook medium, washed twice in PBS, aliquoted in PBS, 10 % glycerol, frozen and stored at -80°C. For infection, aliquots were thawed and bacterial titers were determined. Aliquots were homogenized by repeated transfer through a syringe with a 0.4 mm ∅ needle and appropriately diluted in PBS. Bacteria were injected in a volume of 200 µl PBS into the lateral tail veins of mice.

4.1.5 Generation of murine macrophages and dendritic cells from bone marrow

Tibia and femur of a 6-10 week old mouse were prepared. Femur was cut off above the hip joint and Tibia was cut away at foot joint. Bones were put on ice in complete medium. Remaining tissue was removed from bones and the joints were removed. Bone marrow cells were rinsed out of bones by using a 20 ml syringe with a 0.6-25 mm ∅ needle and complete medium. The cell suspension was centrifuged at 1,500 rpm, 5 min, 4°C and the resuspended pellet was subjected to red blood cell lysis. Cells were counted and maximally $2x10^7$ cells were plated in a non-cell-culture Petri dish in 10 ml complete medium. After incubating the cells for 2h at 37°C, 5% CO_2, the supernatant containing non adherent cells was removed and plated on a fresh Petri dish. GM-CSF (20 ng/ml) was added to these cells to generate DCs. After removal of the supernatant, the remaining, adherent cells were incubated to macrophage medium to culture macrophages. Medium was changed after 3 and 7 days by taking away half of medium and adding fresh medium. DCs will be ready to use after 10-12 days and macrophages will be ready to use after 7 days after isolation. To remove cells from the Petri dishes, remove SN, add cold PBS, incubate 10 min at 4°C, carefully scrape off and wash cells.

4.1.6 Generation of human DCs from peripheral blood

Fifteen ml Biocoll solution was added to a 50 ml conical tube and 35 ml blood was carefully layered on top of it without mixing the solutions. Tubes were centrifuged at 1,900 rpm for 25 min at RT without brake. After centrifugation, a dense layer of PBMCs could be detected in the Biocoll gradient. PBMCs were recovered from the gradient and transferred to a 50 ml tube containing RPMI. Cells were washed and centrifuged at 1500 rpm for 5 min at RT. The cell pellet was resuspended in PBS and centrifuged at 1,200 rpm for 5 min at RT to deplete blood platelets. Remaining cells were treated with erythrocyte lysis buffer to lyse red blood cells. PBMCs were then resuspended in PBS, transferred to a cell culture flask and incubated for 60-90 min at 37°C, 5% CO_2 to let macrophages and monocytes attach. Next, the supernatant was taken away and complete medium for human cells, containing GM-CSF (at 200 U / ml) and IL-4 (at 100 U /ml), was added. Cells were incubated at 37°C, 5% CO_2 to generate immature DCs. Immature DCs are generated at Day 3 after isolation.

4.1.7 Generation and restimulation of human NKT cell lines and clones

Human NKT cell lines and clones were provided by Dr. M. Bonneville (Inserm U601, Institut de Biologie, Nantes, France). Briefly, T cells were stained for TCR Vα24 chain and CD4 expression using standard staining procedures and analyzed by flow cytometry. Vα24$^+$ cells were sorted using streptavidin magnetic beads coated with biotinylated TCR Vα24 antibody, expanded using lectin, IL2 and irradiated feeder cells as described, and analyzed for expression of Vα24 and Vβ11 TCR chains.
For restimulation and expansion, 1x10^6 T cells were incubated with 1x10^6 irradiated, freshly isolated PBMCs, 1x10^6 B-EBV transfected B cells, PHA (at 1mg/ml) and IL-2 (at 300 U/ml) in a volume of 150 µl in a 96 well plate. Cells were pooled and transferred to a cell culture flask with fresh medium containing IL-2 (300 U/ml) when the medium started to turn yellow. T cells were diluted every 2-5 days and cells were used earliest 10 days after stimulation. PBMCs and B-EBV transfected B cells were irradiated with 3,500 rad. B-EBV transfected B cells had been cultured in RPMI medium and PBMCs were isolated as described in chapter 3.1.6

4.1.8 Single cell isolation from different tissues

Spleen cells were isolated by homogenization using an iron mash sieve and subsequent red blood cell lysis using erythrocyte lysis buffer.
Liver cells were isolated by use of a percoll gradient. Briefly, mice were sacrified, cut open and the liver was rinsed with sterile PBS injected through the *vena hepatica*. Liver cells were homogenized using an iron mash sieve and centrifuged at 400 rpm for 2 min at 4°C. Supernatant was collected and the remaining pellet was resuspended in 15 ml RPMI and centrifuged at 400 rpm, 4°C, 2 min. This was repeated 3 times and finally the supernatant from the first centrifugation and the supernatant from the following centrifugations were centrifuged at 1,500 rpm for 10 min at 4°C. Cells were resuspended and further purified over a 40% / 70% Percoll gradient and centrifugation at 1,900 rpm for 30min at RT. Lymphocytes were collected at the interface of the 40% / 70% percoll gradient and collected cells were washed twice in complete RPMI medium, red blood cells were lysed and cells were counted. Viability of the cells was determined by Trypan-blue exclusion.

4.1.9 Red blood cell lysis

After isolation of cells from organs, cells were washed in sterile PBS and resuspended in erythrocyte lysis buffer and incubated at 37°C for 2 min. Erythrocyte lysis was stopped by washing the cells with a 1:1 mixture of PBS and complete medium.

4.1.10 Flow cytometry

To measure surface expressed proteins, 2x10^6 cells were washed and incubated in 200 µl PBS with rat serum and anti-CD16/CD32 mAb for 5 min at 4°C to block non-specific antibody binding. Next, fluorescent dye conjugated antibodies were added to the cells for 20 min on ice in the dark, washed in PBS and resuspended in 300 µl PBS containing 0.1% BSA for flow cytometry analysis.

To measure intracellular cytokines, cells were first stained for surface expressed proteins, then washed in PBS and fixed for 20 min on ice in the dark with PBS with 4% paraformaldehyde (*Sigma*). Cells were washed with PBS with 0.1% BSA, permeabilized with PBS with 0.1% BSA and 0.5% saponin (*Sigma*) and incubated in this buffer with rat serum and anti-CD16/CD32 mAb for 5 min. To stain the intracellular cytokines conjugated or conjugated isotype control mAb were added. After 20 min on ice, cells were washed with PBS and fixed with PBS with 1% paraformaldehyde. Next, cells were washed and resuspended in 300 µl PBS containing 0.1% BSA for flow cytometry analysis.
When using unconjugated primary antibodies, a second staining with a secondary antibody, usually an anti-rat antibody, recognizing the primary antibody, was performed. After the staining with the primary antibody, cells were washed with PBS with 0.1% BSA and excess rat Ig was added to block unspecific binding of the secondary antibody. Cells were incubated for 5 min on ice in the dark and subsequently the secondary antibody was added. Following an incubation time of 15 min on ice in the dark, cells were washed in PBS and resuspended in 300 µl PBS containing 0.1% BSA for flow cytometry analysis.
For staining cells with CD1d-tetramer complexes, $2x10^6$ cells were washed and incubated in 200 µl PBS with rat serum and anti-CD16/CD32 mAb for 5 min at 4°C to block non-specific antibody binding. Next, fluorescent dye conjugated antibodies and PE-conjugated CD1d-tetramers were added to the cells and incubated for 50 min on ice in the dark on a rocking platform. Subsequently cells were washed in PBS and resuspended in 300 µl PBS containing 0.1% BSA for flow cytometry analysis. For flow cytometry staining, 2.5 - 5 µg of tetramerized mCD1d was used.
Detection of viable cells was achieved by staining the cells with PI or DAPI shortly before analysis.
Counting of stained cells was performed with a FACS Calibur or FACS Canto (*Becton Dickinson*) and the software FCS-express and Cell Quest were used to analyze the data.

4.1.11 Isolation and purification of cells by MACS and FACS sorting

Magnetic cell sorting (MACS) with MicroBeads (*Miltenyi*) was performed to enrich cell populations by positive selection. For some applications it was decided to positively select “unwanted” cells and thereby negatively select and enrich the desired cells. Cells were either selected by beads conjugated to a certain surface molecule displayed on their surface, or stained with a fluorescent dye conjugated antibody first and then selected with magnetic beads conjugated with an antibody specifically recognizing the fluorescent dye. Cells were labeled according to the manufacturers protocol (*Miltenyi Biotec MACS MicroBeads*)
After labeling the cells, the cell suspension was loaded on a column which was placed in the magnetic field of a MACS Separator. The magnetically labeled cells were retained in the column while the unlabeled cells passed through. After removal of the column from the magnetic field, the magnetically retained cells could be eluted as the positively selected cell fraction.

Fluorescence activated cell sorting (FACS) was performed at the flow cytometry core facility at the MPI for Infection Biology. Cells were labeled with antibodies and/or tetramers and subsequently sorted using the FACS-sorter FACS-Diva (Becton Dickinson, BD Bioscience). All steps were done on ice and

cells were kept at 4°C at all times. The sorting regions were defined in the dot blot diagrams showing forward (FSC) versus 90° (SSC) scatter signals on a linear scale with cell debris characterized by FSC/SSC signals distinct from that of the intact cell population being excluded from sorting. Viable cells were detected by staining with PI or DAPI and cells were sorted into a tube containing 500 µl cold PBS, 0.1% BSA. The purity of sorted fractions was checked visually and by FACS reanalysis. The FACS-sorter was standardized with fluorochrome-containing beads, and fluorescence-reading in each channel was automatically adjusted to a constant value.

4.1.12 Isolation of lysosomes from fibroblast

Fibroblasts were grown to confluency and cells were scraped of on ice in 5 ml lysis buffer. All steps were carried out on ice. Cells were transferred to a cell douncer and the douncer was applied 20-30 times to crack the cells. The cell suspension was centrifuged at 800 rpm for 10 min at 4°C to obtain the post-nuclear supernatant (PNS). An aliquot of the supernatant was examined for remaining cells and eventually the douncer was applied again till no cells were visible. The PNS was further purified using a 20% percoll gradient in lysis buffer. Four ml of PNS was added carefully to 10 ml of 20% percoll without mixing the solutions. The tubes were centrifuged at 18,000 rpm for 21 min at 4°C without brake and supernatant was discarded leaving 3 ml in each tube. Samples were pooled and ultracentrifuged at 50,000 rpm for 60 min at 4°C. After centrifugation, lysosomes were visible as a small cloud and were recovered from the tubes. Lysosome preparations were washed 2 times by adding 200-300 µl of the lysosome preparation to 1-2 ml PBS and centrifugation at 50,000 rpm for 60 min at 4°C. Lysosomes were recovered from remaining gel-like pellet with a small amount of PBS. Aliquots of the lysosomal preparation were prepared and stored at -80°C. Protein content was determined using the Bradford Protein Assay.

4.1.13 T cell stimulation assay

Spleen cells derived from Vα14-Jα281 transgenic mice were used since they possess three times more NK1.1 positive T cells than wild type C57BL/6 mice and no alloreactivity to the APC (H-2D) was observed as assessed with splenic T cells from C57BL/6 mice. Isolated spleen cells were incubated for 1 h in cell culture flasks to remove adherent cells. To enrich for T cells, magnetic cell separation using anti-Thy1.2 FITC conjugated antibodies and anti-FITC coated magnetic beads were used. Purified T cells were seeded at $1x10^5$ cells/well in 96-well round bottom plates. As APCs, A20-CD1d expressing cells or parental A20 cells, DCs or macrophages of C57BL/6 or $CD1^{-/-}$ mice ($5x10^4$ cells/well) were pulsed with the respective lipids for 3 h at 37°C, irradiated (20,000 rad for A20 cells; 5,000 rad for murine DCs or macrophages) and extensively washed before adding them to the T cells. Cells were incubated for 18-24 h.

For human T cell stimulation assays, NKT cell lines and NKT cell clones, generously provided by Dr. M. Bonneville (Inserm U601, Institut de Biologie, Nantes, France) were used at $1-2x10^5$ per well in a 96-well plate. As APCs, human DCs were generated from healthy donor blood or from blood of

patients with Fabry disease. DCs and macrophages were pulsed with the respective lipids for 3-6 h, irradiated (5,000 rad) and subsequently T cells were added. Cells were incubated for 18-48h.

Cytokines in the cell culture supernatant were detected using ELISA and proliferation of T cells was measured by [3H] thymidine incorporation. Cytokine levels and proliferation are expressed as mean +/- SD of triplicate cultures.
The respective lipids were dissolved in DMSO at a concentration of 10 mg/ml, 1 mg/ml or 100 µg/ml and a range from 10 µg - 100 ng were added to the APCs.
To block the α-GalA activity, APCs were pretreated with Jirimycin (20 µg/ml) for 45 - 60 min prior to addition of lipid antigens. To reconstitute the α-GalA activity in DCs derived from Fabry patients, α-GalA (Replagal 1 mg/ml) was added 30 min prior to the lipid antigens to the APCs.

4.1.14 Dual luciferase reporter assays for NF-κB activation.

The NF-κB dependent luciferase reporter assay was performed as described previously (Aliprantis et al., 1999b). Briefly, HEK293 cells were seeded in 48-well tissue culture plates at a density of 1.5 x10^4 cells/well. After 48 h, cells were transfected by FuGENE (*Roche*) with the expression plasmids pgD-hTLR2 (coding for human TLR2) or empty vector (pRKN) 0.3 µg, 0.03 µg of pGL3-ELAM-tk and 0.003 µg of the Renilla luciferase reporter vector pRL-TK (*Promega*) for normalization. A FuGene / serum free medium (DMEM) and a 3 / 1 ratio of FuGene / DNA was used. The DNA of the different plasmids were mixed, added to the FuGene / DMEM mix and incubated for 15 min at RT. Twenty µl of this mix were added to each well and the plate was gently swirled for even distribution. After 24h, cells were stimulated for 6 h with the bacterial lipopeptides and lipids as indicated and the reporter gene activity was measured according to the manufacturer´s recommendations (*Promega*) using a plate reader luminometer (*BD Biosciences, Luminometer monolight 3036*). The data shown represent 1 of 3 separate experiments and are shown as the mean values ± S.D. of triplicate samples. Results are reported as fold induction of relative luciferase units (RLU) over unstimulated cells. Relative luciferase activity is calculated as the ratio between the value of the NF-κB inducible firefly luciferase and that of the constitutive *Renilla* luciferase reporter.

4.2 Biochemical methods

4.2.1 ELISA

Immuno-Maxisorp ELISA plates (*Nunc*) were coated with the primary antibody in 50 µl/well coating buffer and incubated for 60 min at 37°C. Plates were washed four times using ELISA wash buffer and blocked for 1 h at 37°C in 200 µl/well PBS containing 0.1% BSA. After repeating the washing step, standards and samples were diluted in tissue culture medium, 50 µl added to each well and left for 60 min at 37°C for binding of the cytokine of interest to the primary antibody. Plates were then washed prior to the addition of 50 µl/well of the secondary antibody diluted to the appropriate concentration in PBS / 0.1% BSA and incubated at 37°C for 1 hour. After repeated washing, 50 µl/well streptavidin-conjugated horseradish peroxidase (*Dianova*) diluted 1:1,000 in PBS / 0.1% BSA was added and incubated for 1 hour at 37°C. After further washing, 50 µl of the peroxidase substrate ortho-phenyldiamine (OPD) (*Sigma*) in 50 µl citrate buffer was added. The reaction was allowed to proceed for 5-10 min in the dark before being stopped by addition of 50 µl 20% H_2SO_4. The plate was read by a Spectramax ELISA reader (*Molecular Devices*) and analyzed by Softmaxpro software. Control wells were left untreated with the primary or secondary antibody or filled with medium only. For murine IFNγ ELISA, primary antibody was R4-6A2, used at 2 µg/ml and secondary antibody was XMG1.2-biotin used at 3 µg/ml. The standard recombinant IFNγ was purchased from *R&D* and diluted from 500 U/ml in 2 fold dilutions. The IFNγ ELISA was linear in the range from 5 U/ml to 100 U/ml. For murine IL-4 ELISA, primary antibody was 11B11, used at 1 µg/ml and secondary antibody was BVD6-24G2-biotin used at 2 µg/ml. The standard recombinant IL-4 was purchased from *R&D* and diluted from 5 ng/ml in two fold dilutions. The IL-4 ELISA was linear in the range from 5 U/ml to 100 U/ml.
Detection of human IFNγ and IL-4 was performed with Cytoset ELISA Kits (*Biosource*) according to the manufacturers descriptions. Optical densities were measured at 490 nm.

4.2.2 Detection of [3H] thymidine incorporation

T cell proliferation was detected by measurement of [3H] thymidine incorporation into the DNA of proliferating cells. One to $2x10^5$ cells were incubated with 20 µCi/ml [3H] thymidine in 200 µl RPMI for 6 h. Cells were harvested and proliferation plates were washed 4x, dried and 40 µl Microscint LSC cocktail was added to intensify the detectable radiation. Proliferation was measured by [3H] thymidine detection using a Topcount NXT microplate scintillation counter.

4.2.3 Isolation of lipids from *M. bovis* BCG

Total lipid extracts were obtained by overnight incubation in chloroform/methanol (2:1; v/v). The solution was passed through a filter to remove the bacteria. The remaining cells were incubated in chloroform/methanol (1:2; v/v) and the solution was passed through a filter to remove the bacteria. Extracts were pooled and centrifuged for 10 min at 4,000 rpm. The supernatant was passed through a

filter and the volume of the chloroform/methanol extract was reduced by using a rotational evaporator. Lipids were further purified by liquid chromatography using a silica gel column (*Pharmacia*). The silica column was equilibrated with chloroform and the lipid extract was applied. The ration of lipid extract and amount of silica gel was 1/200. Elution of the different lipid fractions was performed with a gradient with increasing amounts of methanol and decreasing amounts of chloroform. Lipid fractions were analyzed for the desired lipids by SDS-PAGE and silver staining. Selected fractions were further purified by reverse-phase chromatography using C18 Sep-Pack ready-to-use columns and a chloroform to methanol gradient. Obtained fractions were analyzed by thin layer chromatography.
LAM, LM and PIM were isolated from *M. bovis* BCG by Triton X-114 extraction. 10^{11} Bacteria were washed with 0.05 M Tris-HCl pH 7.5 (TBS) and lyophylized. Bacteria were resuspended in TBS, 5mM EDTA and sonicated on ice using a microtip-sonicator. TBS containing $MgCl_2$ (10 mM) was added and proteins and nucleic acids were removed by RNAse/DNAse (1 µg/ml) digestion. Triton X-114 was added (8% v/v) to the crude extract, stirred for 16 h at 4 °C and cellular debris was removed by centrifugation for 30 min at 10,000 x g. After induction of phase separation and centrifugation, the Triton X-114 phase was recovered and the remaining solution was reextracted with the cellular debris using Triton X-114. The resulting Triton fraction were pooled and precipitated with cold ethanol. Precipitated glycolipids were collected by centrifugation at 10,000 x g for 30 min, the pellet was resuspended in ddH_2O, stirred overnight and ultra-centrifuged at 35,000 x g for 16 h. The sediment was resuspended in ddH_2O and treated with Proteinase K (1 mg/ml) for 2 h at 37°C. The solution as ultra-centrifuged and resuspended twice, reconstituted in ddH_2O and lyophylized. Glycolipids were then dissolved in Tris-deoxycholic acid buffer (Tris-HCl 10 mM, pH 8.0; EDTA 10 mM; NaCl 0.2 M; Deoxycholic acid 0.25%; NaN_3, 0.02%) and separated using a 100x1.5 cm column packed with Sephacryl S-200. Lipid fractions were eluted with Tri-deoxycholic acid buffer and the resulting fractions were analyzed by SDS-PAGE and silver staining. Selected fractions were pooled, dialyzed against ddH_2O using dialysis tubes with a molecular cut out of 10,000 Da (LAM and LM) or 1,000 Da (PIM) and lyophylized.

4.2.4 SDS polyacrylamide gel electrophoresis

Lipids fractions, dissolved in DMSO, were separated on a discontinuous SDS-PAGE according to Laemmli using vertical slab gels (Laemmli, 1970). Samples were prepared in SDS-containing sample buffer and heated to 95°C for 3 min before electrophoresis. Lipids were visualized by silver staining.

4.2.5 Silver staining

SDS-PAGE gels were incubated for 1 h in fixing solution on a rocking platform. Proteins and glycolipids contained in the gel were oxidized by addition with 30 mM $NaIO_4$ in for 10 minutes. Gels were then treated for 30 min with a 10% acetic acid, 10% methanol solution and subsequently incubated with 2.5% glutaraldehyde for 5 minutes. Gels were washed 4x with ddH_2O, incubated with 25 µg/ml dithiotreitol for 5 min and rocked for 5 min in ddH_2O with 1 mg/ml silvernitrate. Gels were shortly washed 2x with ddH_2O. To visualize proteins and glycolipids, gels were treated with developer

which was replaced after 10 seconds with fresh solution. The reaction was stopped after 2 min by addition of citric acid (50%) in ddH_2O. The gels were dried between cellophane sheets.

4.2.6 High performance thin layer chromatography (HPTLC) analysis

For two dimensional HPTLC glycolipid samples were spotted in one corner of a 10 x 10 cm HPTLC plate. For one-dimensional HPTLC, glycolipid samples were spotted as a line 1.5 cm above the lower edge of the HPTLC plate. The HPTLC plate was developed to 8 cm and solvents used were: chloroform/methanol/ammonium hydroxide (80:20:2; v/v/v), propanol/water/acetic acid (80:10:10; v/v/v), chloroform/methanol/water (65:25:4; v/v/v) or chloroform/acetic acid/methanol/water (40/25/3/6). Hundred µg lipid was spotted on the plate and lipids were analyzed for sugar residues using 1% α-naphthol in 5% H_2SO_4/ethanol as a spray reagent. Using α-naphthol, carbohydrate residues are stained blue, whereas red staining indicates absence of carbohydrates.

4.2.7 Matrix assisted laser disorption ionization – mass spectrometry (MALDI-MS)

Lipids were analyzed for their structure using MALDI-MS. Fifty µg of lipid sample was dissolved in chloroform and mixed with 10 µl 2, 5-dihydroxybenzoic acid as a matrix. The mixture was dried and analyzed. Laser intensity was set at 1,200 and time of flight was determined in negative mode.
MALDI-MS analysis was performed at the mass-spectrometry core facility of the MPI for Infection-Biology.

4.2.8 Protein quantification by Bradford

Samples were diluted with PBS to an estimated concentration of 1 to 20 µg/ml. A standard dilution row was prepared containing a range of 1 to 20 µg protein (albumin was used) to a volume of 100 µl. One or 5 µl of unknown samples was added to 100 µl H_2O and 900 µl dye reagent (Sigma) was added. The solution was mixed and incubated for 5 min at RT. Absorbance was measured at 595 nm and concentrations were determined by use of the standard dilution row.

4.2.9 Generation of tetrameric mCD1d - lipid complexes

Tetrameric mCD1d complexes were made in a baculovirus expression system as described previously (Crawford et al., 1998).The generation of tetramers was previously described (Matsuda et al., 2000) and the plasmid coding for the recombinant murine CD1d protein was generously provided by Dr. M. Kronenberg (La Jolla Institute for Allergy and Immunology, San Diego, USA).
The CD1d tetramers loaded with different lipid antigens were generated at the biochemistry core facilities of the MPI for Infection Biology. Briefly, the mCD1d/mß2m expression vector was cotransfected with linearized BaculoGold™ baculovirus DNA (*PharMingen*) into High Five™ (BTI-TN-

5B1-4) cells (*Invitrogen*) using the Lipofectin Reagent® (*Gibco BRL*) by following the manufacturer's protocol. Recombinant virus was collected 5 d after transfection, amplified, and cloned by serial dilution method. The virus with highest level of mCD1d secretion was used for protein production. Soluble protein was produced by infecting adherent High Five™ cells at a MOI of 5-10. The mCD1d-containing supernatant was harvested on day 4-5 after the infection, dialyzed against 0.15 M sodium phosphate buffer, pH 7.4, and passed over Ni–agarose (*Qiagen*) for one-step affinity purification. Purified mCD1d protein was biotinylated with BirA enzyme (*Avidity*) following the manufacturer's protocol. The αGalCer-loaded mCD1d was tetramerized by adding neutravidin–PE *(Molecular Probes*) in 4:1 molar ratio. "Unloaded" mCD1d tetramers were prepared by preincubating biotinylated protein with an equivalent amount of αGalCer dilution vehicle.

4.3 Molecular biological methods

The general molecular biological methods like agarose gel preparation, DNA electrophoresis, DNA precipitation and measurement of DNA / RNA concentration were performed according to *Molecular Cloning: A Laboratory Manual* (Sambrook and Russell, 2001). DNA sequences were compared using the web-based program Blast 2 Sequences (http://www.ncbi.nlm.nih.gov/gorf/bl2.html)

4.3.1 Isolation and purification of RNA from single cell suspensions

Total RNA was isolated by the TRIzol® Reagent RNA preparation method (*Gibco*). Briefly, cells were resuspended immediately after FACS sorting in 500 µl TRIzol®, shock frozen and stored at -80°C. Cells were thawed and further processed for total RNA isolation as described by the manufacturer. The amount of RNA was determined by OD260/280nm measurement and total RNA was purified by RNeasy (*Qiagen*). The RNA integrity and the amount of total RNA were measured with a Bioanalyzer 2100 (*Agilent Technologies*).

4.3.2 Generation of cDNA and RT-PCR

Initially purified RNA samples were treated with DNAse (*Gibco*) to eliminate genomic DNA contamination. For this, 8 µg of RNA in 16 µl H_2O and 1 µl of 10x reaction buffer were incubated with 2 µl of DNAse I for 15 min at RT. The reaction was stopped by the addition of 2 µl 25 mM EDTA and the DNAse was inactivated by incubating the mixture at 65°C for 15 min. While 12 µl of this mixture were stored and later analyzed for the completion of the DNAse digestion, 10 µl of it were used for reverse transcription. For this 1 µl random hexamer primers (200 µg/ml) were added, the mixture was incubated for 10 min at 65°C and then placed on ice. After 5 min on ice, a reaction mix containing 4 µl 5x first strand buffer, 1 µl 10 mM dNTPs, 2 µl 0.1 M DTT and 1 µl of superscript reverse transcriptase was added. This mixture was immediately incubated for 5 min at 25°C, followed by 60 min at 50°C and

finally incubated for 15 min at 70°C in order to inactivate the reverse transcriptase and to stop the reaction.

4.3.3 Real-time RT-PCR

Quantification of DNA using real-time PCR is based on the measurement of amplified products after each cycle of the PCR using fluorescent dyes interacting only with double stranded DNA. The more template is present at the beginning of the reaction, the lower the number of cycles it takes to reach a point in which the fluorescent signal is first recorded as statistically significant above background, which is the definition of the threshold cycle. Comparison of the threshold cycle for a specific template in each sample leads to semi-quantitative evaluation of original template concentration. For semi-quantitative real-time PCR total RNA was isolated from cells as described in section 4.3.1 and transcribed to cDNA as described in section 4.3.2. All PCRs were run for 40 cycles with 20 sec 94°C and 60 sec 60°C in the ABI Prism 7900 Sequence Detection System (*Applied Biosystems*) using ABI PRISM optical 96-well plates (*Applied Biosystems*). When possible primers were designed to span large introns and to produce product sizes between 100 and 200 bp. Reaction mixtures were set up in 30 µl final volume using 15 pmol of each primer, 5 µl template cDNA and 15 µl 2x SYBR-Green PCR Master mix (*Applied Biosystems*). For the quantification of GAPDH and β-actin, 5 µl of 1:150 diluted cDNA were used as template in each reaction and for the quantification of e.g. cytokines, 5 µl of 1:15 diluted cDNA were used. Each quantification was performed at least twice with independent cDNA samples and in duplicates for each cDNA and primer pair. Data analysis was performed using the ABI Prism 7000 SDS Software and *Microsoft* Excel. The threshold cycle was determined for each sample and fold differences relative to the expression level in one of the analyzed cDNA samples was calculated for each cDNA sample and primer pair (fold-difference=$2^{-\Delta Ct}$). Resulting fold differences for expression levels were corrected for different amounts of cDNA by multiplication with the average fold difference of GAPDH and β-actin expression within the same sample. The number of cycles necessary to produce enough PCR-product for detection but small enough to not exceed the log phase of the PCR was determined by serial dilutions of cDNA or multiple PCRs with different numbers of cycles. To compare the amount of cDNA used in each reaction, β-actin primers were included. Possible contamination with genomic DNA was estimated using 1:5 diluted DNAse digested, not reverse transcribed, RNA as template.

4.3.4 RNA microarray

Microarray experiments were done as two-color hybridizations. Total RNA was extracted from single cell suspensions as described in section 4.3.1. An amount of 4 µg total RNA was reverse transcribed with an oligo-dT-T7-promotor primer by a fluorescent linear amplification reaction (*Agilent Technologies*) and cDNA was labeled either with Cyanine 3-CTP and Cyanine 5-CTP (*NEB Life Science Products*) in a T7 polymerase amplification reaction according to the supplier's protocol. In order to compensate specific effects of the dyes, e.g. incorporation and to ensure statistically relevant

data analysis, a color swap was performed. The RNA samples were labeled vice versa with the two fluorescent dyes (fluorescence reversal). After precipitation, purification and quantification, 1.25 µg of each labeled cRNA was mixed, fragmented and hybridized to the 8.4 K custom *'in situ'* mouse array according to the supplier`s protocol (*Agilent Technologies*). Scanning of microarrays was performed with 5 µm resolution using a DNA microarray laser scanner (*Agilent Technologies*). Features were extracted with an image analysis tool Version A4.045 from *Agilent Technologies* using default settings. Data analysis was carried out on the Rosetta Inpharmatics platform Resolver Built 3.0.0.3.22.

5 Results

5.1 Characterization of NKT cells

NKT cells influence and regulate a wide range of immune responses. They play a role in autoimmunity, allergy, antitumour responses, host defence to infections and in inflammation (Godfrey and Kronenberg, 2004). NKT cells show high similarities to other cells including NK cells, $CD4^+CD25^-$ (conventional $CD4^+$ T cells) T cells and $CD4^+CD25^+$ T cells (regulatory T cells, Treg). Nevertheless, NKT cells possess unique features that distinguish them from other cells. In order to characterize NKT cells on the transcriptome level and to analyse their functional capabilities, gene expression profiling using RNA microarray technology was performed.

5.1.1 Transcriptome analysis of NKT cells compared with NK cells, conventional $CD4^+$ T cells and Treg cells

To examine cell-type specific characteristics of NKT cells, microarray studies were designed comparing NKT cells to other T cells and NK cells. Experiments were performed using RNA extracted from spleen cells of C57BL/6 mice. RNA from naïve, unstimulated NKT cells was isolated and compared to RNA from naïve, unstimulated NK cells, conventional $CD4^+$ T cells and Treg cells. In order to obtain RNA from highly pure and viable cells, cells were stained with antibodies for their characteristic surface markers. Subsequently cells were purified and enriched by MACS-sorting prior to FACS-sorting. To isolate NK cells, cells were stained with an APC-conjugated anti-CD3 and a FITC-conjugated anti-NK1.1 antibody. Subsequently, they were enriched by positive MACS sorting using anti-FITC magnetic beads selecting for NK1.1 positive ($NK1.1^+$) cells. NK cells were sorted by FACS sorting as $NK1.1^+$ and $CD3^-$ cells. To sort NKT cells, spleen cells were stained with FITC-conjugated anti-NK1.1 antibody and NKT cells were enriched by MACS sorting with anti-FITC beads. Positively selected cells were then stained with APC-conjugated anti-CD3 and PE-conjugated αGalCer-CD1d tetramer. NKT cells were isolated as $CD3^+$, $NK1.1^+$ and αGalCer-CD1d $tetramer^+$ cells. These cells will be referred to as NKT cells during the whole work.

Surprisingly, microarray results showed a number of genes known to be expressed by macrophages, suggesting minute contaminations of macrophages in the sorted NKT cell population. This was unexpected since reanalysis of the sorted samples showed a purity of >95 % due to stringent sorting parameters and a blocking step was included to prevent unspecific antibody binding. To improve the sorting protocol further, the following steps were added: to enrich NKT cells and deplete macrophages, B cells, $CD8^+$ T cells and dendritic

cells with high efficacy, spleen cells were stained with biotin-conjugated antibodies against Mac-1, B220, CD11c and CD8. Magnetic beads, coated with anti-biotin antibody were used to remove macrophages, B cells, DCs and $CD8^+$ T cells. The non-binding cells (MACS-negative) were further processed. Cells were stained with anti-CD3, anti-NK1.1 antibodies and streptavidin-PE-Cy7 was used to deplete left over cells from the positive depleted cells. NKT cells were sorted as $CD3^+$, $NK1.1^+$, αGalCer-CD1d $tetramer^+$ and streptavidin-PE-Cy7-negative, i.e. Mac-1-, B220-, CD11c-, CD8- negative cells (Figure 7). FACS analysis of purified cells revealed that NK cells were 99 % pure and NKT cells were > 90 % pure.

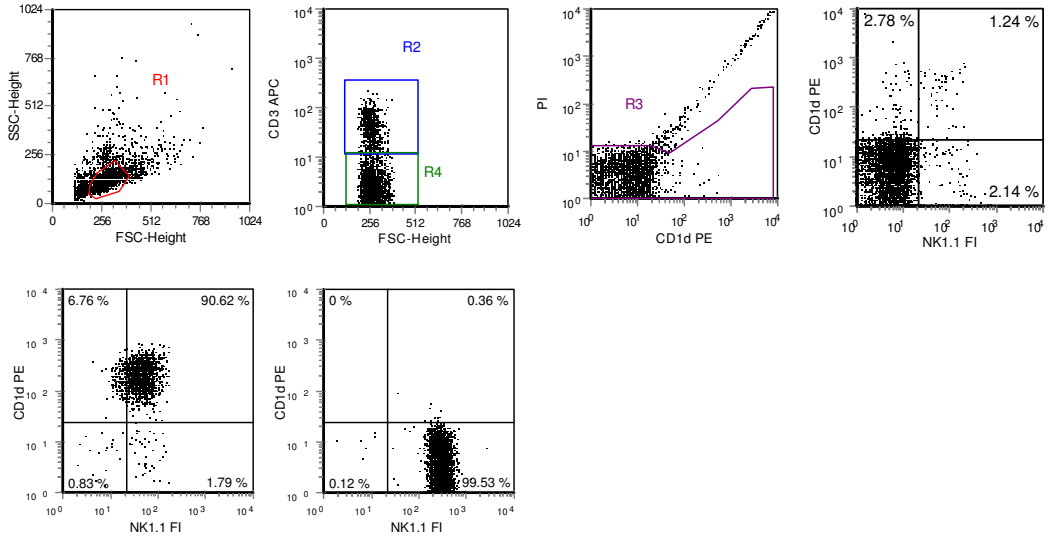


Figure **7**: FACS sort of murine NK and NKT cells from C75BL/6 mice. Upper row: unsorted spleen cells with sorting gates and dot blot of unsorted NKT cells. Lower row: (left) sorted NKT cells and (right) sorted NK cells. Cells were stained with antibodies against CD3 and NK1.1 and CD1d-αGalCer tetramers. NKT cells were sorted as $CD3^+$, $Nk1.1^+$ and $tetramer^+$ cells. NK cells were sorted as $CD3^-$, $NK1.1^+$and $tetramer^-$ cells. Percentages of positive cells are indicated in the quadrants. Dead cells were excluded by PI staining and only viable cells were sorted.

Conventional $CD4^+$ T cells and Treg cells were isolated from C57BL/6 mouse spleen cells by staining for CD4, CD25 and NK1.1. Cells were stained with an APC-conjugated anti-CD25, a FITC-conjugated anti-CD4 and a PE-conjugated NK1.1 antibody. Cells were enriched by positive MACS-sorting using anti-FITC magnetic beads selecting only for $CD4^+$ cells. Both CD4 T cell populations were sorted as $NK1.1^-$ and either $CD25^+$ or $CD25^-$, CD4 expressing T cells. Conventional $CD4^+$ T cells were 98 % pure and Treg cells were 97 % pure (Figure 8).

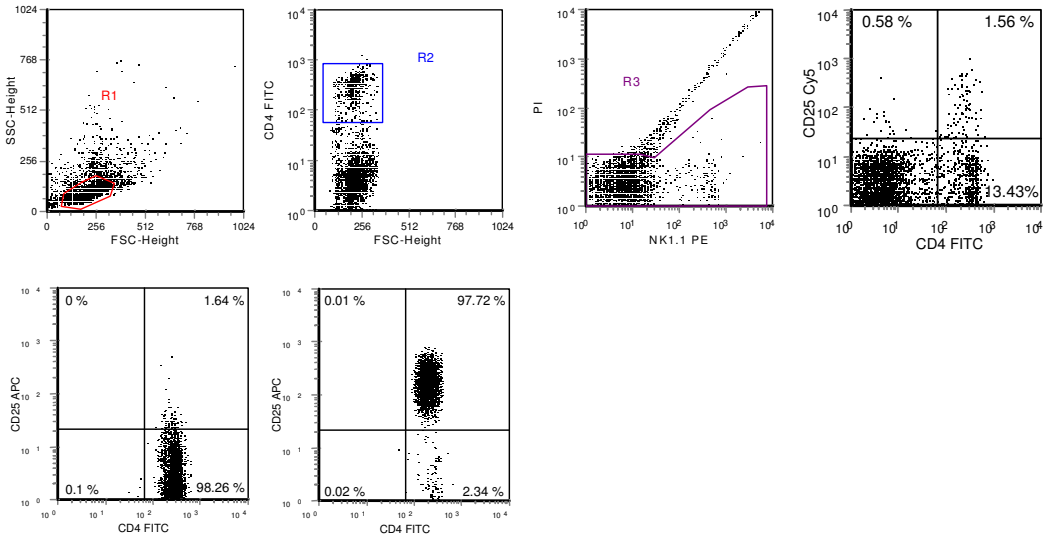


Figure **8**: FACS sort of murine CD4+ T cells from C75BL/6 mice. Upper row: unsorted spleen cells with sorting gates and dot blot of unsorted T cells. Lower row: (left) sorted $CD4^+$ $CD25^-$ T cells and (right) sorted $CD4^+CD25^+$ T cells. Cells were stained with antibodies against CD3, CD4 and CD25. T cells were sorted as $CD3^+$, $CD4^+$ and $CD25^-$ or $CD25^+$ cells. Percentages of positive cells are indicated in the quadrants. Dead cells were excluded by PI staining and only viable cells were sorted.

In all cell purifications, PI or DAPI was added shortly before sorting the cells to discriminate between viable and dead cells. Only PI / DAPI negative, i.e. viable cells were collected. Cells were maintained on ice, enriched by MACS at 4°C and sorted into tubes containing cold PBS, 0.1% BSA to avoid activation of the cells. At least $5x10^5$ cells were isolated by FACS sorting and further used for microarray analysis. FACS sorts were repeated 4 times and duplicate samples of each sorted cell type were used for two independent microarray studies.

After sorting NK cells, conventional $CD4^+$ T cells, Treg cells and NKT cells, cells were centrifuged and resuspended in TRIzol® to avoid RNA degradation and samples were stored in liquid nitrogen. Cells were thawed and further processed for total RNA isolation as described in section 4.3.1. The amount of RNA was determined by OD260/280nm measurement and total RNA was purified by an RNA isolation kit (RNeasy; *Qiagen*). The RNA integrity and the amount of total RNA was measured with a Bioanalyzer 2100 (*Agilent Technologies*) (Figure 9).

RNA was purified and analyzed from all sorted populations and characteristic bands for 28s and 18s RNA were detected. RNA amounts were in the range of 6 ng/µl to 175 ng/µl. The RNA of the isolated cell populations were labeled with Cy5 or Cy3 and

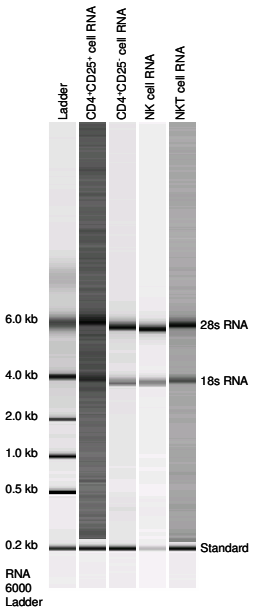


Figure **9**: RNA isolated from FACS sorted cells of C57BL/6 mice. RNA bands for 28s and 18s RNA are marked.

hybridized to Agilent custom made microarrays that contained oligonucleotide probes representing 8013 different gene sequences predicted to be involved in immunologic processes. The hybridization was repeated on a second array with the technique of “color swap” of the dye-labels to exclude artefacts due to labeling effects and to minimize errors. After measurement of spot-color and intensity on both arrays, data were analyzed using the Rosetta Inpharmatics Resolver software. Significantly up- and downregulated genes were selected for each cell type by correlating the ratio between the two colors of each measured sample from the first array with the ratio of the same sample in the second array where the dy-colors were swapped (Figure 10).

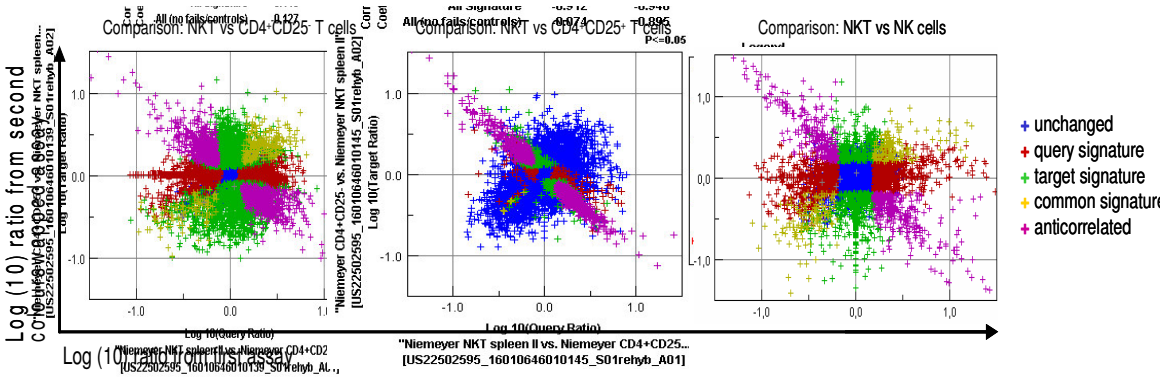


Figure **10**: Correlation blots of Log(10) ratios deducted from 2 independent hybridizations of Cy5 and Cy3 labeled RNA isolated from NKT cells, NK cells, $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells. Significantly different ratios were anticorrelated between the 2 hybridizations and are depicted in purple. Ratios depicted in blue were unchanged between both hybridizations and red or green ratios were only present in 1 of the 2 hybridizations.

Only anticorrelated genes were considered to be differentially regulated in the analysis of gene expression of the different cell types. Namely, those genes that were Cy5 positive in the first array and Cy3 positive with a similar intensity in the second array. The Resolver software calculates the probability value from a variety of internal controls, including the degree of anticorrelation for each measured ratio. This P value reflects the reliability of the measured data in the same way as P values for significances do.

To exclude erroneous detection of genes and to make microarray results more reliable, two independent microarray analyses were performed. For the first comparative analysis, RNA from NKT cells, according to the initial sorting parameters, was used. For the second analysis, RNA of cells, depleted of B cells, macrophages, DCs and $CD8^+$ T cells was used. NKT cell RNA was used as reference RNA, i.e. gene expression levels found in other cells were related to the levels detected in NKT cells. Therefore, genes are considered upregulated when their expression level is increased compared to NKT cells and downregulated when the expression level is lower in other cells, i.e. upregulated in NKT

cells. Only genes with P values <0,05 and a minimum of 2 fold difference are considered to be significantly regulated.

In the first microarray analysis, 778 genes were found to be differentially regulated between NKT cells and NK cells, conventional $CD4^+$ T cells and Treg cells (Figure 11). Of these, 121 genes were found higher or lower expressed within all cell types compared to NKT cells. Four hundred-thirteen genes were detected to be upregulated in other cells, i.e. downregulated in NKT cells. Amongst these 413 genes, 10 genes showed an increased expression in all other cell types compared to NKT cells.
A union of 440 genes with decreased expression levels in NK cells, conventional $CD4^+$ T cells or Treg cells, i.e. higher expression in NKT cells, was detected. Sixty-two 62 of these genes were downregulated in NK cells, conventional $CD4^+$ T cells and Treg cells, describing a set of genes, uniquely upregulated in NKT cells.

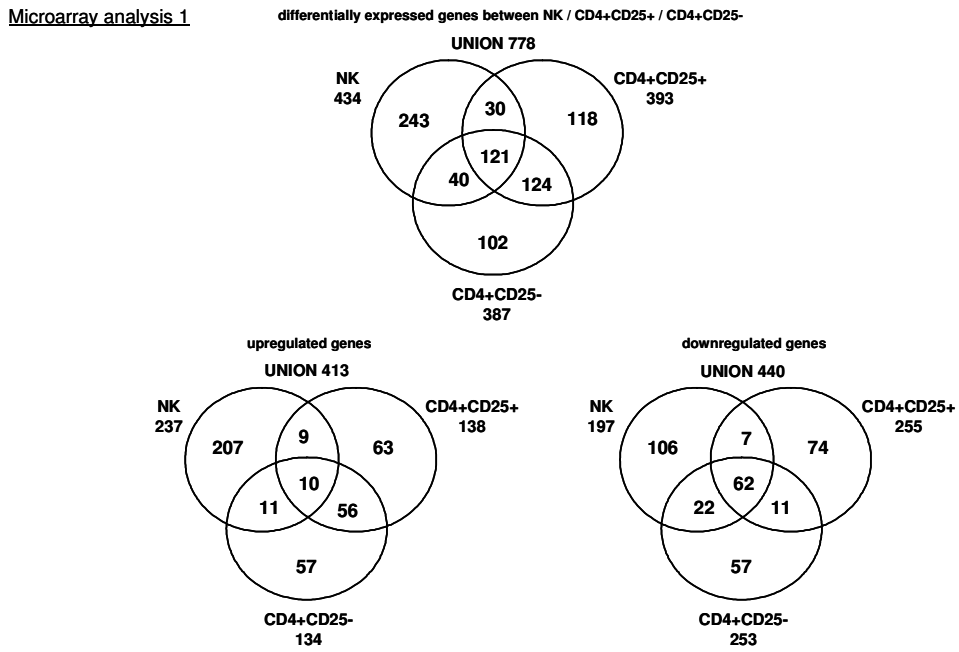


Figure 11: Venn diagram of microarray analysis of NKT cells compared with NK cells, CD4+CD25- and CD4+CD25+ cells from spleen of C57BL/6 mice. NKT cell RNA was used as the basis for gene expression profiling. The figure summarizes genes that are differentially expressed in the cell types described. Upregulated genes: upregulated in all other cell types compared to NKT cells. Downregulated genes: downregulated in all other cell types compared to NKT cells, i.e. upregulated genes in NKT cells. Numbers indicate the number of genes. Union: Number of all genes regulated.

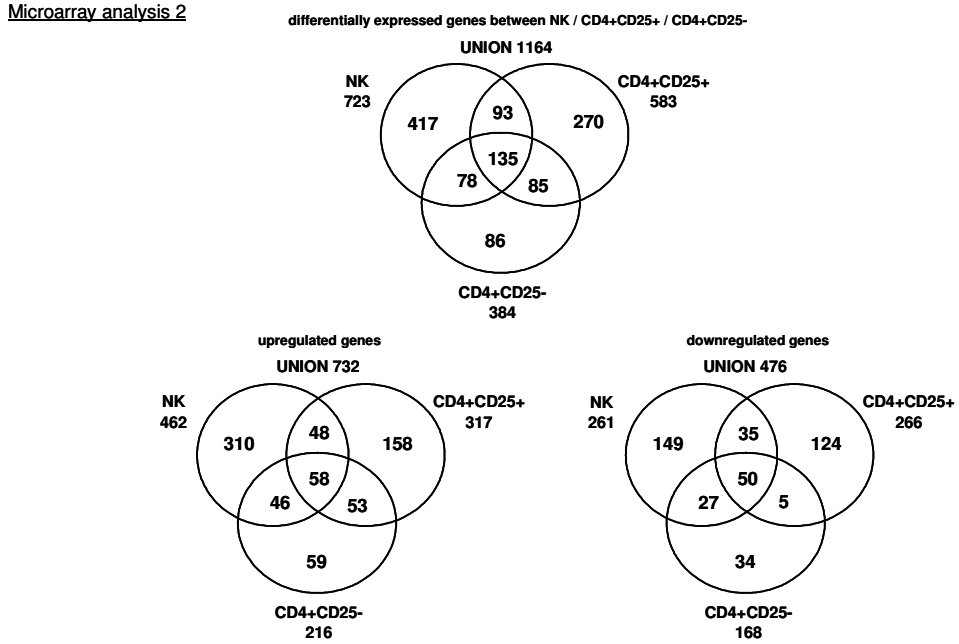


Figure 11 continued: Venn diagram of microarray analysis of NKT cells compared with NK cells, CD4+CD25- and CD4+CD25+ cells from spleen of C57BL/6 mice. NKT cell RNA was used as the basis for gene expression profiling. The figure summarizes genes that are differentially expressed in the cell types described. Upregulated genes: upregulated in all other cell types compared to NKT cells. Downregulated genes: downregulated in all other cell types compared to NKT cells, i.e. upregulated genes in NKT cells. Numbers indicate the number of genes. Union: Number of all genes regulated.

The second microarray analysis detected 1,164 genes differentially expressed between the analyzed cell types with 135 genes up- or downregulated collectively within compared populations. Seven hundred thirty-two genes with higher expression in NK cells, conventional $CD4^+$ T cells or Treg cells were identified. Of these, 58 genes showed increased expression in all cell types compared to NKT cells. The number of genes with decreased expression in NK cells, conventional $CD4^+$ T cells or Treg cells compared to NKT cells was 476 with 50 genes being downregulated in NKT cells (Figure 11).Only genes up- or downregulated in both microarray comparisons were considered as verified using two independent microarray analyses. These genes were used for further investigation.

5.1.2 Unique gene expression of NKT cells

Two hundred thirty-eight genes, containing 107 ESTs, were found with increased expression in both arrays in NK cells, conventional $CD4^+$ T cells or Treg cells compared to NKT cells. One hundred ninety-two genes were detected with a decreased expression in conventional $CD4^+$ T cells or Treg cells. Eighty-seven of these genes were ESTs. Only 6 genes were upregulated in all cell types in both arrays, i.e. downregulated in NKT cells. In comparison,

20 genes with lower expression levels in all other cells compared to NKT cells were identified in both arrays. These genes describe a group of genes upregulated uniquely in NKT cells.

Genes with lower expression levels in NK cells, conventional $CD4^+$ T cells and Treg cells were of major interest. They resemble genes that could be exclusively expressed in NKT cells or show a NKT cell specific expression pattern. A high proportion of these genes code for cytokines and chemokines or cytokine- and chemokine-receptors. Some of these genes were already described in the context of NKT cells, like IL-12 receptor β1, CXCR3 and integrin-α4β1 (VLA-4) (Franitza et al., 2004; Kim et al., 2002; Kitamura et al., 1999). The IL-12 receptor-β1 binds to IL-12 which is a pro-inflammatory cytokine (Zhang et al., 2003) and CXCR3 binds to interferon-inducible CXC chemokines IP-10, MIG, and I-TAC and is a chemokine receptor for homing to non-lymphoid tissue (Tamaru et al., 1998; Xie et al., 2003). Integrin-α4β1 is involved in lymphopoiesis and inflammatory recruitment of leukocytes (Rivera-Nieves et al., 2005). The other genes, significantly expressed by NKT cells have not previously been attributed to this T cell subset. Among these genes are BLK, CCR3, DAO1, IL-17 receptor B and LIGHT. BLK belongs to the family of Bcl-2 proteins, which regulate programmed cell death. BLK is pro-apoptotic and essential for initiation of programmed cell death and stress-induced apoptosis (Coultas et al., 2004; Hegde et al., 1998). BLK was found in hematopoietic cell types but so far not associated with NKT cells. Microarray analysis detected BLK as 11 fold, 5 fold and 3 fold upregulated in NKT cells compared to all other cell types. The chemokine receptor CCR3 is expressed on eosinophils and Th2 cells and binds a variety of chemokines including RANTES and eotaxin (Lamkhioued et al., 2003). It was detected with a 14 fold higher expression in NKT cells compared to NK cells, 2.5 fold to conventional $CD4^+$ T cells and 5 fold to Treg cells. The IL-17 receptor-B binds IL-17 which is produced by T cells after activation. IL-17 has pro-inflammatory capacities both directly and by amplifying effects of IL-1β and TNFα and is able to induce production of pro-inflammatory cytokines. IL-17 can also induce matrix metalloproteinases (MMPs) (Stamp et al., 2004). The IL-17 receptor-B was 15 fold, 7 fold and 7 fold upregulated in NKT cells compared to NK cells, conventional $CD4^+$ T cells and Treg cells, respectively. Further genes were DAO1, which performs oxidative deamination of neutral D-amino acids and whose function for T cell activity is not known (Konno et al., 1993). LIGHT is a member of the TNF family with co-stimulatory capacities in the context of TCR signaling (Shi et al., 2002). MMP9 is important in tissue remodeling, recruitment of leukocytes to sites of inflammation and in processing of cytokines (Odaka et al., 2005).RORA is a member of the steroid hormone nuclear receptor superfamily and can bind melatonin which activates IL-2 production (Guerrero et al., 2000).

Genes with decreased expression levels in NKT cells included caspase 1, caspase 11, integrin- α4β1, L-selectin, KLF2 and vimentin. L-selectin showed the strongest downregulation with 13 fold, 10 fold and 10 fold in NKT cells compared to NK cells, conventional $CD4^+$ T cells and Treg cells, respectively (Table 2).

Table **2**: Genes uniquely regulated in NKT cells

Fold difference between mRNA levels of NK cells, $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells vs. NKT cells			**Sequence name**	**NCBI Accession number**	**Gene**
NK cells	**$CD4^+CD25^-$**	**$CD4^+CD25^+$**			
-10.54	-4.77	-3.05	Biklk	NM_007546	Bcl2-interacting killer-like = Blk
-13.88	-2.46	-4.99	Cmkbr1l2	NM_009914	chemokine receptor 3 (CCR3)
-3.20	-4.09	-3.33	Cmkar3	NM_009910	chemokine receptor 3 (CXCR3)
-7.32	-2.30	-4.63	Dao1	NM_010018	D-amino acid oxidase = DAO1
-14.69	-6.96	-7.32	Il17br	NM_019583	IL-17 receptor B
-3.48	-3.12	-6.43	Il12rb1	NM_008353	interleukin 12 receptor-β1
-2.10	-2.08	-4.07	Tnfsf14	NM_019418	LIGHT
-5.47	-3.80	-5.74	Mmp9	NM_013599	matrix metalloproteinase 9 (MMP-9)
-7.16	-3.09	-3.41	AW045613	AW045613	RORA / RZR = RAR-related orphan receptor α
8.71	2.63	2.87	Casp1	NM_009807	caspase 1
4.46	3.17	3.30	Casp11	NM_007609	caspase 11
7.81	2.54	3.22	Itga4	NM_010576	Integrin-α4β1 / VLA-4
4.44	3.54	3.94	Klf2	NM_008452	Kruppel-like factor 2 (KLF2)
13.37	9.86	9.73	Sell	NM_011346	L-selectin
6.04	5.03	3.72	Vim	NM_011701	vimentin

Genes that were up and downregulated in NKT cells compared to NK cells, conventional $CD4^+$ T cells and Treg cells are shown. Data were obtained by microarray analysis as described in section 3.3.4. Depicted are: fold differences of genes found in 2 independent microarray analysis, sequence name, NCBI accession number and name of the gene. Genes in black color are downregulated uniquely in all cell types compared to NKT cells, i.e. upregulated in NKT cells. Genes depicted in blue are upregulated uniquely in all cell types compared to NKT cells, i.e. downregulated in NKT cells. Only genes with P values <0.05 and a minimum of 2 fold difference are shown

Caspase 1 cleaves the pro-inflammatory cytokines pro-IL-1β and pro-IL-18 to mature cytokines and has pro-apoptotic capacities (Shi, 2002; Pirhonen et al., 1999). Caspase 11 is a mediator of septic shock response and induced in most cells upon pro-inflammatory stimuli (Kang et al., 2002). Integrin-α4β1 and L-selectin are both important in cell migration. Integrin-α4β1 plays a role in lymphopoiesis and inflammatory recruitment of leukocytes (Rivera-Nieves et al., 2005). L-selectin is important for trafficking of lymphocytes to lymph nodes (Galkina et al., 2003). Vimentin is required for lymphocyte rigidity and involved in transendothelial migration (Brown et al., 2001), whereas KLF2, a transcription factor involved in cell regulation during inflammation is also anti-proliferative and a pro-survival factor (Lin et al., 2003; SenBanerjee et al., 2004; Wu and Lingrel, 2004). Genes in table 2 depict the most

prominent genes up- or downregulated in NK cells, conventional CD4$^+$ T cells and Treg cells compared with NKT cell expression levels. These genes describe what could be a unique gene expression profile of NKT cells; either displaying genes only expressed in NKT cells or being significantly more up- or downregulated in comparison to other cells.

5.1.3 Genes with expression levels shared by NK cells, conventional CD4$^+$ T cells or Treg cells and NKT cells

Table 3 depicts some of the most prominent and interesting genes found with an increased or decreased expression in NKT cells in comparison to one or more cell types examined in these assays. These genes represent those which are not unique for NKT cells but are rather shared with NK cells, conventional CD4$^+$ T cells and Treg cells. For instance, genes with high expression levels in NKT cells and NK cells, but not in CD4$^+$ T cells are attributable to innate immunity-like properties of NKT cells.

Genes upregulated in NK cells, but downregulated in CD4$^+$ T cells compared to NKT cells were: Annexin A1, RANTES, CCL6 and NKG2D. Genes similarly expressed in NK and NKT cells but with clearly lower expression to CD4$^+$ T cells were: ATAC, CCR2, CXCR4, FasL, LFA-1 (integrin-αL), IFNγ and L-CCR. Similar expression levels for NK and NKT cells, but increased expression in CD4$^+$ T cells were detected for: CD30, integrin-α6 and OX40. Comparable gene expression between NKT cells and CD4$^+$ T cells was found for the following genes: CD6, IGTP, IL-4 receptor, IL-7 receptor and IL-15. With the exception of IL-15, which was upregulated in NK cells over NKT cells, CD6, IGT, IL-4 receptor and IL-7 receptor were similarly expressed in NKT cells and CD4$^+$ T cells but downregulated in NK cells. Type II IL-1 receptor expression was increased in conventional CD4$^+$ T cells and unchanged in NK cells and Treg cells. Upregulation in Treg cells and similar expression in NK cells and conventional CD4$^+$ T cells compared to NKT cells was found for the IL-2 receptor gene. TLR4 was expressed stronger in NK cells, similar in conventional CD4$^+$ T cells and less in Treg cells.

Expression of lymphotactin, CCL5, CCR2, CXCR4, FasL, LFA-1, IFNγ, NKG2D and granzyme B were already described for NKT cells (Emoto et al., 1999a; Faunce and Stein-Streilein, 2002; Gumperz et al., 2002; Kennedy et al., 2000; Kim et al., 2002; Leite-de-Moraes et al., 2000; Matsuda et al., 2002; Metelitsa et al., 2003). Expression of CD30 was described for a NKT cell lymphoma (Tao et al., 2002).

Expression of IL-2 receptor, IL-4 receptor and IL-7 receptor has not been specifically demonstrated for NKT cells. However, IL-2 receptor is expressed on activated T and B cells as well as on NK cells (Smith, 1989) and IL-4 receptor is expressed on hemopoietic and nonhemopoietic cells (Lowenthal et al., 1988). IL-7 receptor is important for T-cell development from hematopoietic stem cells and present in thymocytes (Akashi et al., 1998; Di Santo and Rodewald, 1998).

Table **3**: Gene expression levels of NK cells, $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells compared to NKT cells

Fold difference between mRNA levels of NK cells, $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells vs. NKT cells			**Sequence name**	**NCBI Accession number**	**Gene**
NK cells	**$CD4^+CD25^-$**	**$CD4^+CD25^+$**			
3.77	-3.96	-5.65	Anxa1	NM_010730	annexin A1
0.60	-8.97	-10.40	Scyc1	NM_008510	ATAC / Lymphotactin
5.10	-10.55	-5.55	Scya5	NM_013653	CCL5 / RANTES
3.81	-5.95	-8.83	Scya6	NM_009139	C10 / CCL6
-6.27	1.50	1.63	Cd6	NM_009852	CD6
1.59	-4.41	-6.38	Cmkbr2	NM_009915	chemokine receptor 2 (CCR2)
1.28	-2.94	-2.02	Sdfr1	NM_009145	CXCR4 (stromal cell derived factor receptor 1)
-1.03	-12.45	-7.06	Tnfsf6	NM_010177	Fas ligand (FasL)
-0.02	-3.59	-2.44	Itgal	M60778	LFA-1 (integrin-αL)
1.33	-4.89	-2.47	Ifng	M28621	IFNγ
-2.92	-0.03	-1.08	Igtp	NM_018738	IFNγ induced GTPase (IGTP)
-1.12	-1.29	-2.73	Il2rb	NM_008368	interleukin 2 receptor
-2.84	0.09	0.18	Il4ra	NM_010557	interleukin 4 receptor
-6.04	-1.59	0.15	Il7r	NM_008372	interleukin 7 receptor
-1.27	-2.07	-4.17	L-CCR	NM_017466	L-CCR (LPS inducible C-C chemokine receptor)
5.04	-5.49	-4.85	D6H12S2489E	AF039026	NKG2D
0.68	3.20	6.72	Tnfsf8	NM_009403	CD30
10.49	1.85	-2.12	Gzmb	NM_013542	granzyme B
0.20	2.85	3.38	Itga6	NM_008397	Integrin-α6
-1.77	5.29	1.64	AV224004	AV224004	Interleukin 1 receptor, type II
4.02	-0.56	-0.10	Il15	NM_008357	interleukin 15
0.60	5.79	2.19	Tnfrsf4	NM_011659	OX40 / CD134
5.28	-1.67	-2.40	Tlr4	NM_021297	toll-like receptor 4

Genes that were up and downregulated in NKT cells compared to NK cells, conventional $CD4^+$ T cells or Treg cells are shown. Data were obtained by microarray analysis as described in section 3.3.4. Depicted are: fold differences of genes found in 2 independent microarray analysis, sequence name, NCBI accession number and name of the gene. Negative values show downregulation in the respective cell type compared to NKT cells and positive values upregulation, i.e. downregulation in NKT cells. Only genes with P values <0.05 are shown and only genes with a minimum of 2 fold difference in expression levels are considered to be regulated.

Genes, thus far not attributed to NKT cells are annexin A1, CCL6, CD6, IGTP, L-CCR, integrin-α6, IL-1 receptor, type II and OX40. OX40 is a co-stimulatory molecule and necessary for ongoing cell survival after antigen stimulation (Bansal-Pakala et al., 2004). Annexin A1 is an inflammatory mediator which acts in an autocrine/paracrine way to limit leukocyte infiltration into inflamed tissue sites (Perretti and Gavins, 2003). CCL6 is a chemotactic agent for monocytes, macrophages and, to a lesser extent, $CD4^+$ T cells and

eosinophils (Berger et al., 1996). The LPS-inducible CC chemokine receptor (L-CCR) is an orphan chemokine receptor that was recently identified in macrophages and could be important in the early phase to inflammation (Oostendorp et al., 2004). CD6 is a co-stimulatory protein for immature thymocytes and could promote thymocyte survival (Singer 2002). IL-1 receptor, type II is a decoy receptor for it's ligand IL-1β and lacks the cytoplasmatic signaling part. Thus, expression reduces IL-1β signaling (Dower et al., 1994). IGTP is a member of the 47kDa GTPase protein family and probably involved in defense against bacterial infection (Butcher et al., 2005; Kaiser et al., 2004) whereas Integrin-α6 is important in cell adhesion and migration (Chung et al., 2004).

Microarray results were confirmed by RT-PCR for the following genes: BLK, CCR3, IL-17 receptor B, KL2, L-selectin, ATAC, FasL and CCL5.

Furthermore, FACS analysis was performed to examine expression of genes, upregulated in NKT cells. Vα14Jα18 transgenic (tg) mice were used since they possess approximately 3 times more NKT cells than C57BL/6 mice, thus fascilitating FACS analysis of NKT cells. Spleen cells were stained with anti-NK1.1, anti-CD3, anti-CD4, anti-CD25, anti-CD11a (LFA-1), anti-FasL, anti-CXCR4 antibodies and αGalCer-CD1d tetramer. Surface expression of LFA-1, FasL and CXCR4 was analyzed.

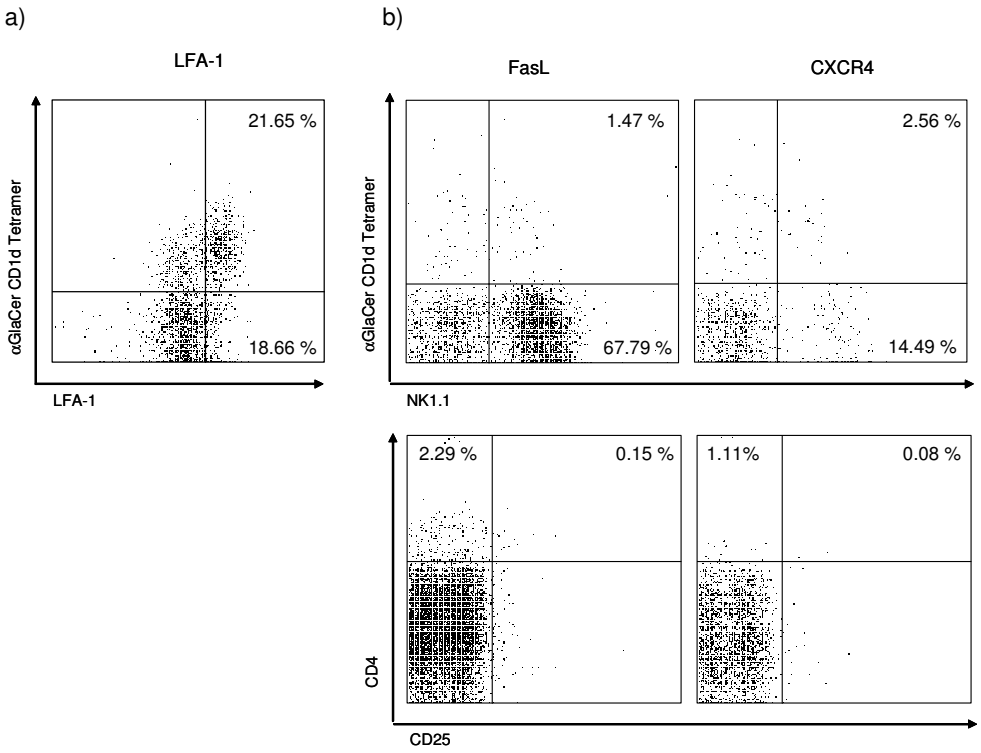


Figure **12**: Spleen NKT and CD4$^+$ cells of Vα14Jα18 tg mice were stained for surface expression of LFA-1, FasL and CXCR4. NKT cells were detected with antibodies against NK1.1 and CD3 and with αGalCer-CD1d tetramer. CD4$^+$ T cells were detected with anti-CD4 and anti-CD25 antibodies. a) Spleen cells gated for NK1.1 expression and analyzed for expression of LFA-1 and for the invariant NKT cell TCR. b) Spleen cells gated for the expression of FasL or CXCR4. FasL and CXCR4 expressing NKT cells were detected with anti-NK1.1 antibody and αGalCer-CD1d tetramer (upper row). FasL and CXCR4 expressing CD4 T cells were detected by anti-CD4 and anti-CD25 antibody (lower row). Percentages of positive cells are indicated in the quadrants. Dead cells were excluded by PI staining and only viable cells were sorted.

Twenty-one % of NKT cells expressed LFA-1 on their surface and 1.47 % were positive for FasL expression. Moreover, 2.56 % NKT cells were stained positive for CXCR4 expression. In comparison, 0.15 % Treg cells and 2.29 % conventional $CD4^+$ T cells were positive for FasL expression. CXCR4 cell surface expression was detected for 1.11 % conventional $CD4^+$ T cells and for 0.08% Treg cells (Figure 12). Thus, data obtained by microarray analysis could be verified by FACS analysis.

5.1.4 Transcriptome analysis of naïve NKT cells vs. activated NKT cells

NKT cells recognize and get activated by lipid antigens presented in the context of CD1d. NKT cells fulfill a variety of diverse functions, however it is not known what mechanisms underlie NKT cell activation and which genes are expressed upon activation.

To detect genes, expressed in activated NKT cells and to define an expression profile, RNA of activated NKT cells was compared to RNA from naïve, non-activated NKT cells. To specifically activate NKT cells 2 µg/mouse of αGalCer was injected i.v. into the base of the tail of C57BL/6 mice. Since NKT cells get rapidly activated after αGalCer administration mice were sacrificed 1 h after injection (Fujii et al., 2003; Lisbonne et al., 2004). Splenic NKT cells were purified as described above by depletion of B cells, macrophages, DCs and $CD8^+$ T cells by MACS. Subsequently, NKT cells were positively selected using FACS sorting. The purity of the sorted populations was confirmed by FACS analysis. Activated NKT cells were ≥ 91 % and naïve, non-stimulated NKT cells were ≥ 92 % pure. RNA was isolated, analysed for 28s and 18s RNA peaks and quantified. The amount of RNA was in the range of 10 ng/µl for activated and naïve NKT cells. The RNA of isolated cell populations were labeled with Cy5 or Cy3, respectively and hybridized to Agilent custom made microarrays, as described above. Data were analyzed using the Rosetta Inpharmatics Resolver software. Significantly up- and downregulated genes were selected for naïve and activated NKT cells by correlating the ratio between the two colors of each measured sample from one array with the ratio of the same sample in the colorswaped second array. In order to verify transcriptome analysis, injection of αGalCer i.v. into C57BL/6 mice was repeated, RNA was isolated and

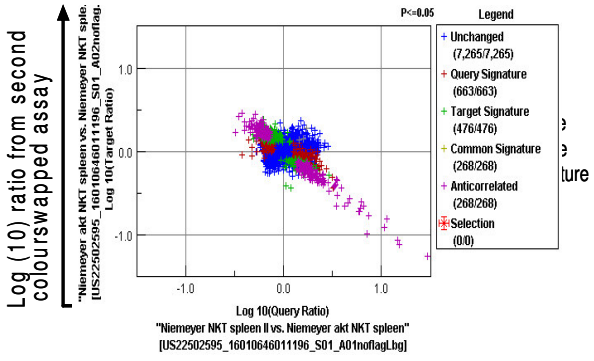


Figure **13**: Correlation blots of Log(10) ratios deducted from 2 independent hybridizations of Cy5 and Cy3 labeled RNA isolated from naïve and activated NKT cells. Significantly different ratios were anticorrelated between the 2 hybridizations and are depicted in purple. Ratios depicted in blue were unchanged between both hybridizations and red or green ratios were only present in one of the two hybridizations

microarray analysis was performed to compare the 2 microarray analysis with each other. Only genes differentially regulated in both arrays were further used to analyze the gene expression profile of activated vs. naïve NKT cells (Figure 13). RNA of naïve NKT cells was used as reference to analyze gene expression of activated NKT cells.

One hundred forty-two genes were found in total in the different arrays with 38 genes upregulated and 3 genes downregulated in αGalCer-activated NKT cells in both comparisons (Table 4). Interleukin 16, MHC class I (H-2^K) and NKR-P1C showed 2 fold lower expression in activated NKT cells. IL-16 is a T cell chemotactic factor and protects against apoptosis (Wilson et al., 2004). MHC class I is involved in antigen presentation (Kaufmann and Schaible, 2005; Lehner and Cresswell, 2004). NKR-P1C is a marker for NK and NKT cells, also known as NK1.1, and downregulation is known for NKT cells (Ljutic et al., 2005; Wilson et al., 2003).

Table **4**: Genes upregulated in activated NKT cells

Fold difference between mRNA levels of naïve and activated NKT cells	Sequence name	NCBI Accession number	Gene
4.04	Scyc1	NM_008510	ATAC / Lymphotactin
18.48	Egr1	NM_007913	early growth response 1 transcriptional factor (EGR1)
8.05	Csf2	X03019	GM-CSF
15.53	Ifng	M28621	IFNγ
28.66	Il13	NM_008355	interleukin 13
3.37	Il17	NM_010552	interleukin 17
4.83	Il2	NM_008366	interleukin 2
9.70	Il4	NM_021283	interleukin 4
2.48	L-CCR	NM_017466	L-CCR
4.00	Tnfsf14	NM_019418	LIGHT
4.55	Scya3	NM_011337	MIP-1 α
7.98	Scya4	NM_013652	MIP-1 β
4.45	Map3k8	NM_007746	mitogen activated protein kinase kinase kinase 8 = Map_3K_8
6.89	Myd118	NM_008655	MyD118 / Gadd45
5.70	Myc	NM_010849	myelocytomatosis oncogene c-Myc
5.86	Nr4a1	NM_010444	nuclear receptor/immediate-early gene Nurr77
12.05	Tnf	NM_013693	tumour necrosis factor / TNF
-2.04	Il16	NM_010551	interleukin 16
-2.27	LOC56628	NM_019909	MHC class I antigen (H-2K)
-2.15	Ly55c	NM_008527	NK1.1 / NKR-P1C

Genes that were up and downregulated upon i.v. injection of αGalCer to stimulate NKT cells are shown. Genes were upregulated in splenic C57BL/6 mice NKT cells. Data were obtained by microarray analysis as described in section 4.3.4. Depicted are fold differences of genes found in 2 independent microarray analyses, name of the gene and NCBI accession number. Genes depicted in black are upregulated, genes depicted in blue are downregulated in activated NKT cells. Only genes with P values <0.05 are shown.

Genes with increased expression levels can be classified into two groups. One group consists of cytokines and chemokines with immuno-stimulating and -regulation properties. IFNγ, IL-13 and TNF were the most significantly upregulated genes, showing 15 fold, 28 fold and 12 fold stronger gene expression in activated NKT cells, respectively. GM-CSF was detected with an 8 fold increased expression and IL-4 exhibited a 9 fold upregulation. ATAC and IL-17 exhibited a 4 fold and 3 fold increased expression, respectively. GM-CSF is a major growth and differentiation factor and activates cells of the immune system to react on infections (Krutzik et al., 2005; Zhang et al., 1999). IL-4 is the main Th2 cytokine (Olofsson, 1991). ATAC is a chemokine attractant mainly produced by $CD8^+$ T cells and NK cells (Dorner et al., 2002). IL-17 is mainly produced by $CD4^+$ T cells and induces production of IL-6 and IL-8 (Stamp et al., 2004). Moreover, genes for IL-2, L-CCR, LIGHT, MIP-1α and MIP-1β showed stronger expression levels in activated NKT cells. The other group of genes upregulated in NKT cells after stimulation is composed of signal transduction molecules, transcription factors and early response genes. EGR1 (early growth response 1 transcriptional factor) is rapidly induced by after activation to transduce the proliferative signal and is anti-apoptotic (Adamson and Mercola, 2002). It showed 18 fold upregulated in activated NKT cells. Map_3K_8, which is part of the Map Kinase signaling pathway triggered by growth factors and cytokines (Beinke et al., 2004), exhibits a 4 fold increased expression. Gadd45, (MyD118), is anti-apoptotic, involved in growth arrest and cell cycle control (Mak and Kultz, 2004) and was upregulated 7 fold. Moreover, c-Myc, expressed in proliferating cells and important in controlling proliferation, differentiation and apoptosis (Brunner and Martin, 2004) had 6 fold increased expression levels. Nurr77 was detected with a 6 fold higher expression. It is rapidly induced by various stress stimuli, including TNF and involved in regulation of apoptosis (Rajpal et al., 2003) (Table 4).

RT-PCR was performed for IFNγ, IL-13, IL-17 to validate results from gene expression profiling of activated NKT cells.

FACS analysis was performed to detect TNFα, IL-4 and IFNγ production by NKT cells after stimulation. Two µg/mouse of αGalCer was injected i.v. into the base of the tail of Vα14Jα18 tg mice. After 2 h, mice were sacrificed and spleens were removed. Spleen cells were analyzed for intracellular expression of TNFα, IFNγ and IL-4. Cells were stained with anti-NK1.1, anti-TNFα, anti-IFNγ, anti-IL-4 antibodies and αGalCer-CD1d tetramer (Figure 14). To analyze NKT cells for TNFα expression, spleen cells were gated for TNFα expression. TNFα-positive cells were analyzed for surface coexpression of NK1.1 and the NKT cell TCR. Nine % of naïve NKT cells were positive for TNFα production. In contrast, activation by αGalCer increased TNFα producing NKT cell numbers to 14.97 % (Figure 14). IFNγ and IL-4

production by NKT cells was detected by gating spleen cells for NK1.1 expression. NKT cells were identified by αGalCer-CD1d tetramer and analyzed for intracellular expression of IFNγ or IL-4. Thirteen % naïve NKT cells were positive for IFNγ and 10.99 % for IL-4 showing strong IFNγ and IL-4 presence in naïve NKT cells. Following 2 h of αGalCer treatment, 30.89 % NKT cells were positive for IFNγ and 25.18 % for IL-4. FACS analysis of TNFα, IL-4 and IFNγ in naïve and activated NKT cells showed elevated levels of these cytokines even in naïve mice. After 2 h of stimulation with αGalCer, strong induction of these cytokines was detected by intracellular cytokine staining (Figure 14).

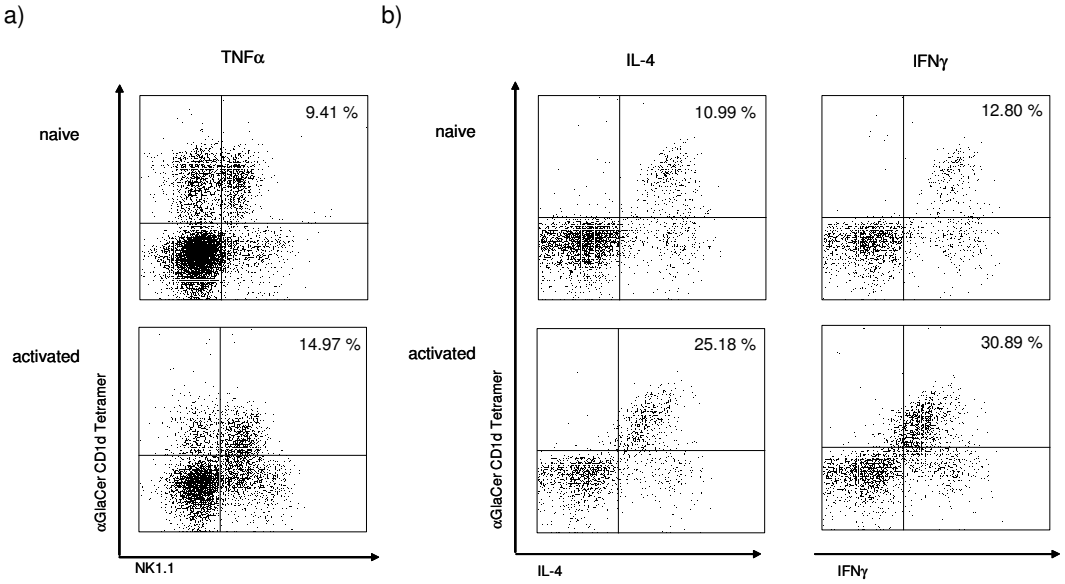


Figure **14**: Spleen NKT cells of naïve and αGalCer treated Vα14Jα18 tg mice were stained for intracellular expression of TNFα, IFNγ and IL-4. NKT cells were detected with antibody against NK1.1 and with αGalCer-CD1d tetramer. a) Spleen cells gated for TNFα expression and analyzed for expression of NK1.1 and invariant NKT cell TCR. b) Spleen cells gated for the expression of NK1.1. IFNγ and IL-4 expressing NKT cells were detected with antibodies against IFNγ or IL-4 and αGalCer-CD1d tetramer. Upper row: spleen cells of naïve mice; lower row: spleen cells of αGalCer treated mice. Percentages of positive cells are indicated in the quadrants. Dead cells were excluded by PI staining and only viable cells were sorted.

Results obtained from microarray analysis indicate that NKT cells possess the ability to respond to and produce various cytokines and respond strongly and rapidly on TCR stimulation.

5.2 Endogenous NKT cell lipid ligands

The best described antigen for NKT cells is αGalCer, a glycosphingolipid derived from the marine sponge *Agelas mauritianus*. It is a surrogate antigen with unclear physiological relevance. In most studies of NKT cell function, NKT cells have been stimulated with αGalCer and investigated by αGalCer-CD1d tetramers. The galactose moiety of αGalCer is attached by an α-glycosidic linkage at C_1 of the galactose to the ceramide moiety (Figure 15). This α-configuration is rarely found in mammalian organisms. A terminal α1→3 linked galactose or n-acetyl-galactose sugar residue were detected for oligosaccharide antigens such as blood groups A and B, the xeno-antigen, the Forssman antigen, and the iso-globoside 3 (iGb3) glycolipid (Heissigerova et al., 2003). Most mammalian glycolipids consist of β-linkages between glucose or galactose residues and the lipid part of glycolipids.

a)

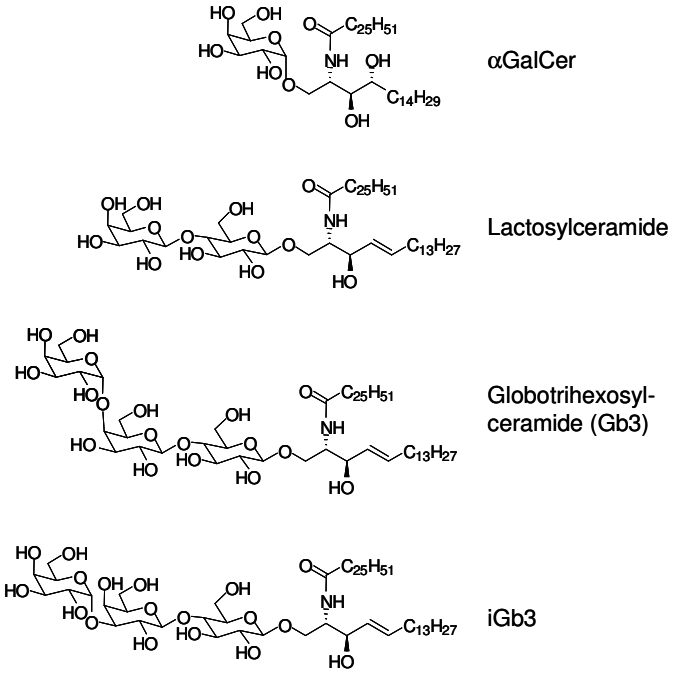


Figure **15**: a) Structures of αGalCer, Gb3, iGb3 and lactosylceramide.

Mature NKT cells display a low-level autoreactivity suggesting the presence of an unknown endogenous CD1d restricted antigen presented continuously on the surface of APCs (Brigl et al., 2003). Globotrihexosylceramide (Gb3), a neutral glycosphingolipid of the globoside series possesses structural similarity to αGalCer in terms of the α-configuration of the sugar-lipid linkage. Gb3 is ubiquitous in mammalian organisms. It is a cleavage product of Gb4, degraded within in the lysosome by αGalactosidase A (αGalA) finally yielding galactose and lactosylceramid.

To test whether Gb3 is the endogenous antigen which is recognized by NKT cells and leads to TCR mediated activation, T cell stimulation assays were performed. Murine A20 cells transfected with CD1d and parental A20 cells, were incubated with αGalCer, ConA, Gb3 and lactosylceramide. After 3 h, NKT cells from Vα14Jα18 tg mice were added and cytokines were measured after 24 h. Lipids were dissolved in DMSO and DMSO alone was used as

negative control. αGalCer and ConA were used as positive control antigens since they induce strong production of IFNγ and IL-4 by NKT cells (Figure 16). DMSO and lactosylceramide did not elicit induction of NKT cells measurable by IFNγ or IL-4 production (Figure 16).
This was expected, since lactosylceramide lacks the terminal galactose in α-configuration. However, Gb3 could not stimulate primary murine NKT cells (Figure 16) This result shows that Gb3 is not recognized by NKT cells and is therefore not the endogenous ligand.

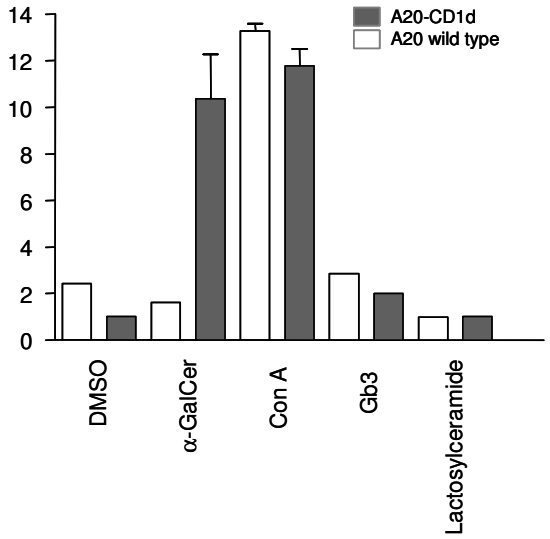


Figure **16**: IFNγ production of murine NKT cells of Vα14 tg mice incubated with different antigens at 1 µg/ml. A20-CD1d and A20 APCs were loaded with lipids, splenic T cells from Vα14Jα281 tg mice were added, and IFNγ was determined 24 h later. Data are expressed as the mean IFNγ concentration, +/- SD of triplicates. One of three experiments is shown.

5.2.1 iGb3: the endogenous NKT cell antigen

iGb3, the isoform of Gb3, was recently described as the endogenous NKT cell ligand. iGb3 elicits a potent NKT cell response and is necessary for NKT cell development (Zhou et al., 2004). Gb3 and iGb3 differ only in the position that the terminal galactose residue is attached to. Gb4 possesses a terminal 1→4 linked galactose, whereas the terminal galactose of iGb3 is attached in a 1→3 linkage (Figure 15).
To examine NKT cell responses to iGb3 more closely, αGalA deficient cells, deficient in iGb3 degradation, from patients with Fabry disease were compared to cells from healthy donors. Blood samples from Fabry patients were obtained in collaboration with Professor Dr. Michael Beck, University Children's Hospital of the University of Mainz.
DCs derived from PBMCs of Fabry patients and healthy donors were incubated with a polyclonal human NKT cell line. This experimental set up provides the opportunity to closely monitor DC - NKT cell interactions and examine effects of antigen availability and control of NKT cell activation. Immature DCs were generated from blood of Fabry patients or healthy donors. DCs were incubated either with iGb3 (10 µg/ml) or with CD40L, for 5 h to see whether DCs are able to present iGb3 and stimulate NKT cells. CD40L induces maturation of immature DCs to mature DCs, leading to an increased presentation of antigens on the cell surface (Fujii et al., 2004). NKT cells from a polyclonal human NKT cell line were added and

incubated for 24 h. Proliferation and cytokine production was measured by [3H] thymidine incorporation and ELISA, respectively.

iGb3 presented by DCs from healthy individuals induced substantial IFNγ and IL-4 production (Figure 17) and proliferation. CD40L-matured DCs did not induce significantly more cytokine production (Figure 17) than the DMSO negative control. Rather, Fabry DCs failed to provoke iGb3 induced production of cytokines by NKT cells (Figure 17). However, addition of CD40L caused an increase in IFNγ production by NKT cells compared to DMSO. αGalCer and ConA were used as positive controls. The lack of cytokine production by iGb3 in Fabry patient derived DCs could not be explained at this point. Nevertheless, this result indicates a mechanism of NKT cell activation-control by antigen availability. CD40L induces maturation of immature DCs leading to increased antigen presentation, which supports this finding. CD40L mediated increase of IFNγ production was only detectable when DCs from Fabry patients were used. It should be noted that iGb3 induced cytokine production, in the presence of APCs generated from healthy individuals, was quite variable between different donors. Nevertheless, availability of antigen and antigen presentation appears crucial for the control of NKT cell activation.

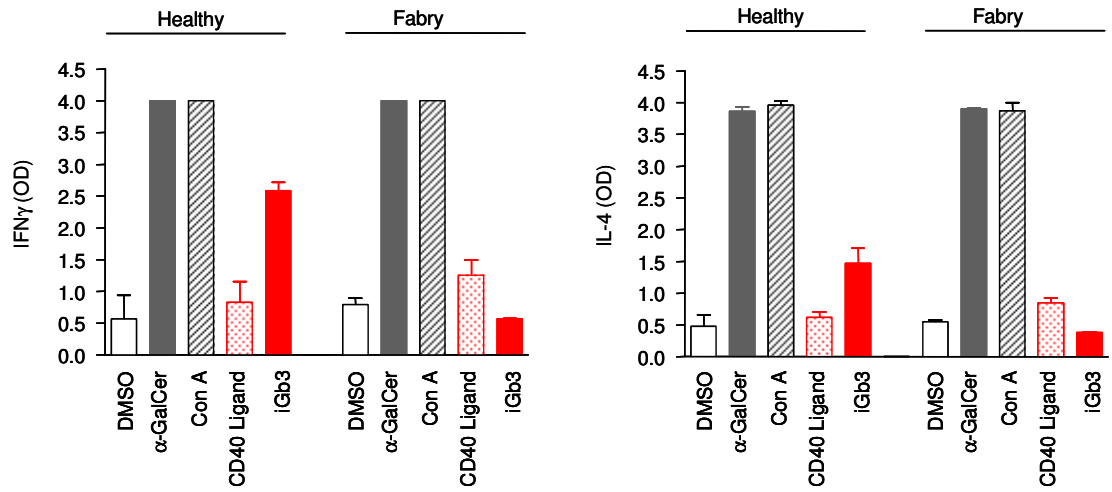


Figure **17**: Cytokine production of a human Vα24 NKT cell line stimulated with different antigens or CD40L in the presence of irradiated DCs as APCs. DCs were derived from blood of healthy donors or Fabry patients as indicated. Cytokines were measured by ELISA after 24 h. CD40L was added to immature DCs for 6h after incubation with antigen for 3 h and prior to addition of NKT cells. Data are expressed as the mean IFNγ concentration, +/- SD of triplicates. One of 3 experiments is shown.

5.2.2 iGb3 and iGb3 analogues

Professor Dr. Peng George Wang, University of Detroit, who initially provided us with iGb3, chemically synthesized iGb3 analogues to test lipid antigens structurally related to iGb3 for their antigenic capacity. Furthermore, experiments were designed to elucidate the question of NKT cell activation-control in more detail. The purity of all synthesized compounds was confirmed by NMR and mass-spectrometry analysis.

PW15 varies from iGb3 by displaying two saturated acyl chains, whereas iGb3 has a double bond in one acyl chain. PW16 is a precursor of αGlycosylceramide (αGlcCer) and PW17 is composed of the sugar moiety of iGb3 and the ceramide part of αGalCer. PW18 is structurally identical with PW16 but possesses a β-configuration connecting sugar to lipid moiety, thus being a βGlcCer precursor (Figure 18).

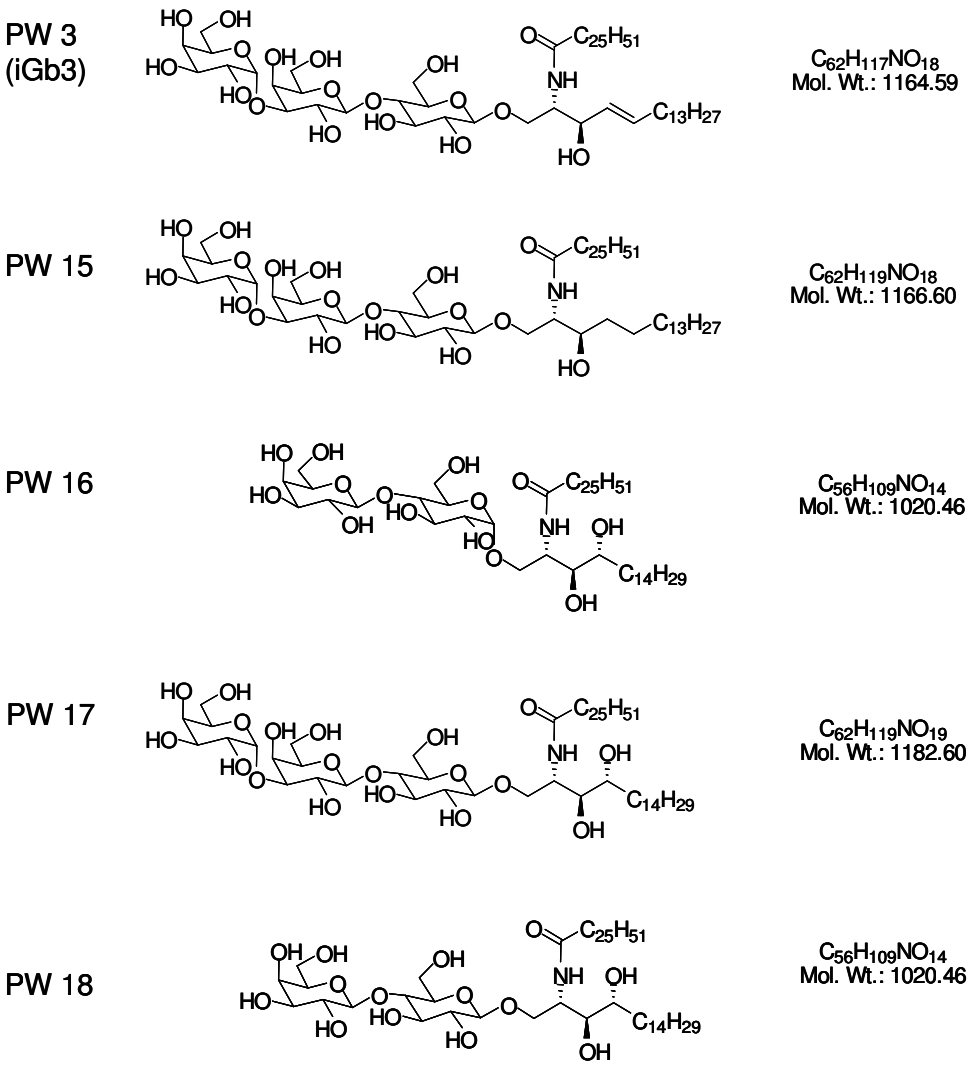


Figure **18**: Structures of iGb3 (PW3) and analogues.

DCs from healthy donors were incubated with iGb3, PW15, PW16, PW17 and PW18 for 6 h. APCs were additionally preincubated for 1 h either with or without Jirmiycin, which specifically blocks αGalA.

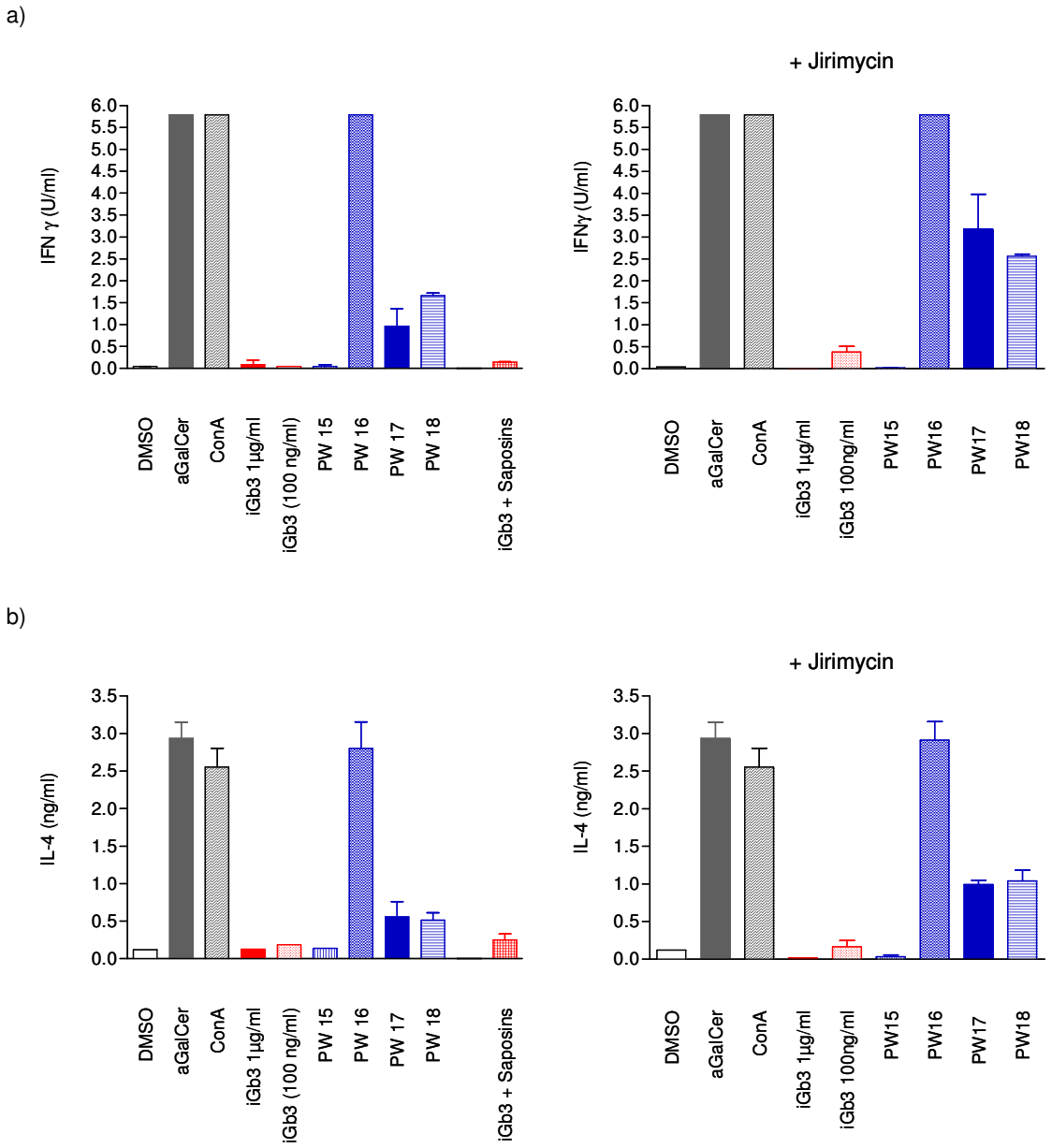


Figure **19**: Cytokine production of a human Vα24 NKT cell line stimulated with different antigens in the presence of irradiated DCs.1x10^5 DCs and 1x10^5 NKT cells were used. Antigens were used at 1 µg/ml unless differently stated. Cytokines were measured by ELISA after 24 h. a) IFNγ production in the absence (left graph) or presence (right graph) of Jirimycin. b) IL-4 production in the absence (left graph) or presence (right graph) of Jirimycin. DCs were derived from blood of healthy donors and Fabry Patients. Jirimycin (20 µg/ml) was used to specifically block the the enzyme α-Galactosidase A; Saposins (Mix of Saposins A and C; 30 µg/ml). Data are expressed as the mean IFNγ concentration, +/- SD of triplicates

Furthermore, a mixture of saposin A and C was added to one well of APCs incubated with iGb3 1 h prior to antigen addition. Saposins are lysosomal proteins which mobilize lipids from lysosomal membranes and facilitate their association with CD1 molecules (Kang and

Cresswell, 2004; Winau et al., 2004). Human NKT cells from a polyclonal NKT cell line were added and culture supernatant was removed after 24 h and analyzed for IFNγ and IL-4 by ELISA. DMSO was used as the negative control and αGalCer and ConA as the positive controls (Figure 19).

In contrast to previous observations, iGb3 used at concentrations of 1 µg/ml or 10 µg/ml did not induce IFNγ or IL-4 production by NKT cells. This discrepancy cannot be explained. PW16 and PW18 induced substantial cytokine production with PW16 being more potent than PW18.

This result proved that DCs processed the antigens from their precursor structure to their biological active form, for surface presentation and NKT cell induction. PW17 induced low IFNγ and IL-4 production whereas PW15, similar to iGb3, did not stimulate NKT cells. Addition of saposins did not have any effect. Addition of Jirimycin led to an increase in cytokine production by NKT cell for PW17 and PW18. PW17 induced significantly more IFNγ and IL-4 in the presence of jirimycin. Jirimycin also enhanced PW18 induced IFNγ but not IL-4 production (Figure 19). The increase in cytokine production induced by PW17 in the presence of jirimycin strongly correlates with the hypothesis that NKT cell activation is critically dependent on antigen availability.

To measure the capacity of iGb3 and iGb3 analogues to selectively expand human NKT cells, PBMCs from healthy donors and Fabry patients were incubated with αGalCer, ConA, iGb3 and iGb3 analogues at different concentrations. After 4 days, PBMCs were analyzed and NKT cell numbers were examined by FACS analysis (Figure 20). Incubation of PBMCs with lipid antigens induced T cell stimulation as detected by the increase in numbers of T cell blastocysts (Figure 20 a). An increase of the NKT cell population was detected upon the incubation with PW17, iGb3 and αGalCer but not in the absence of antigen. Incubation of healthy donor PBMCs with αGalCer, ConA, PW17 and iGb3 induced a striking increase in NKT cell numbers (Figure 20 b). The optimal concentration of lipid antigens to stimulate NKT cells is 100 ng/ml (Sidobre et al., 2002). This concentration induced a strong increase in NKT cell numbers. Upon αGalCer stimulation, 19.49 % NKT cells were detected and 18.86 % NKT cells upon iGb3 stimulation. In the absence of antigen, 10.19 % NKT cells were detected. Both lipids were used at 100 ng/ml. Higher concentrations of iGb3, αGalCer or PW17 (1 - 10 µg/ml) induced strong NKT cell responses (Figure 20 b). PBMCs isolated from Fabry patients, showed a strikingly different response. ConA which is an unspecific T cell stimulus, and used as CD1d independent positive control, still increased T cell numbers to 22.57 % compared with 12 % in the absence of antigen. After αGalCer-treatment, 11.7 %

NKT cells were detected and 12.36 % NKT cells after iGb3 stimulation (Figure 20 c). Thus, incubation of PBMCs from Fabry patients with αGalCer and iGb3, exhibited a lack of NKT cell response compared to healthy PBMCs. Even more striking differences were observed when Fabry PBMCs were incubated with higher concentrations of antigen. One µg/ml iGb3 or αGalCer lead to a reduction of NKT cells to 8.43 % or 9.5 %, respectively. Treatment with 10 µg/ml of iGb3 or PW17 caused a dramatic reduction of NKT cell numbers to 1.25 % (PW17) and 1.4 % (iGb3) (Figure 20 c).

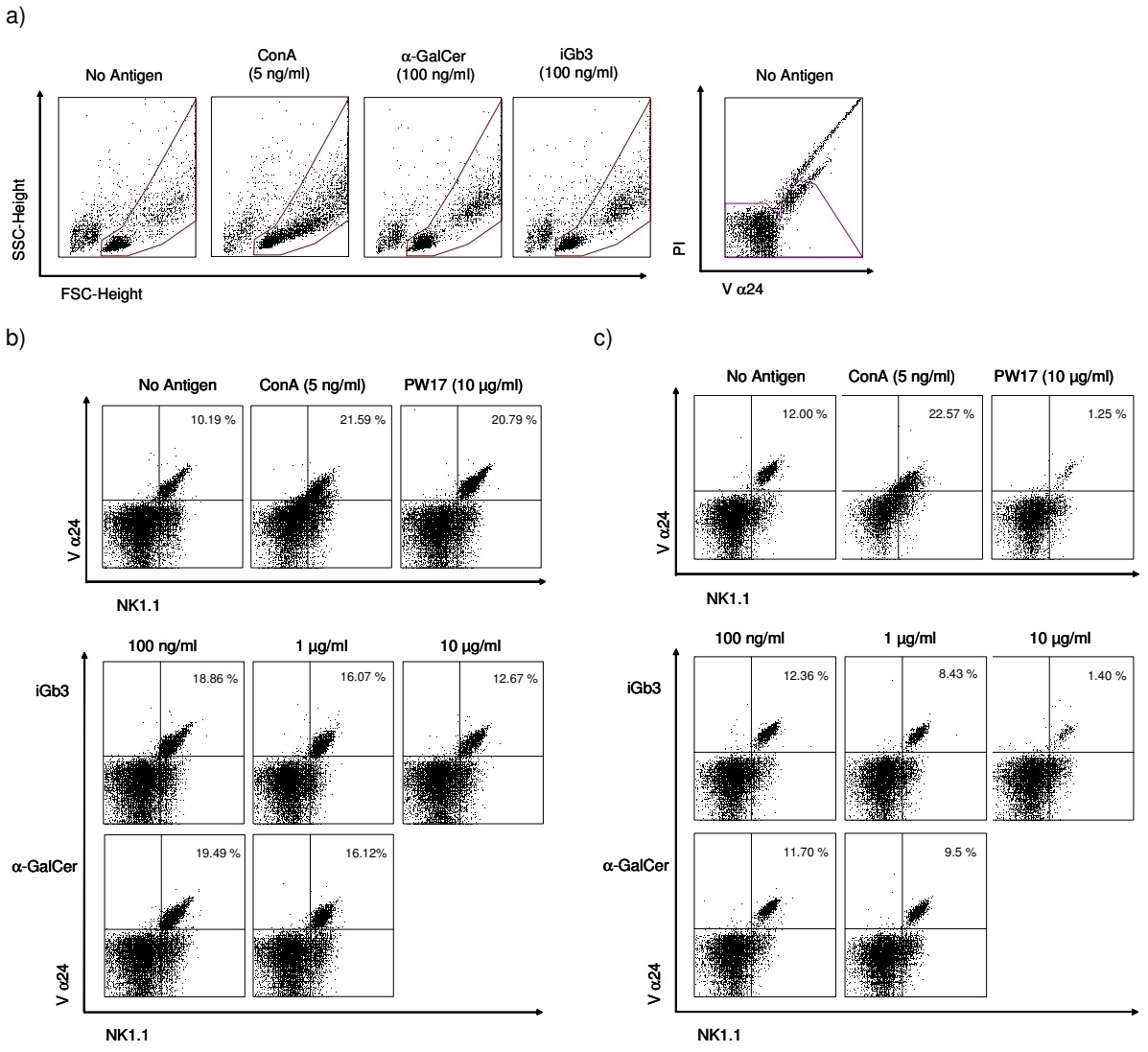


Figure **20**: FACS analyses of human Vα24 NKT cells. a) (left) FACS blots of NKT cells analyzed by their morphology. Activated T cells exhibit a different morphology detected by a shift on the FSC parameter axis. Regions for analysing PBLs are shown. (right) PI staining to exclude dead cells. B) Frequency of hVα24 NKT PBL from healthy donors. c) Frequency of hVα24 NKT PBL from Fabry patients. NKT cells were double-stained with antibodies against NK1.1 and Vα24 TCR chain. PBMCs ($1x10^6$) were cultured for 4 d with different lipid antigens or medium alone, as indicated. Percentages of positive cells are indicated in the quadrants. Dead cells were excluded by PI staining. One of 3 experiments is shown.

These results suggest that a deficiency in lysosomal processing and degradation, leading to an accumulation of NKT cell ligands, has a critical impact in the regulation of NKT cell activation.

5.3 Mycobacterial PIM is a natural NKT cell ligand

A characteristic feature of NKT cells is the recognition of lipid antigens presented by APCs through CD1d. The marine sponge-derived lipid αGalCer, in the context of CD1d, is a potent stimulator of all Vα14Jα281 T cell receptor (TCR) expressing NKT cells in mice and their cognates in man expressing a Vα24JαQ TCR. For years, αGalCer was the only known ligand for NKT cells and bacterial lipids recognized by NKT cells were not identified. Mycobacteria possess a unique, lipid rich cell wall and shed lipids while residing in phagosomes of macrophages (Fischer et al., 2001). The question was whether mycobacterial cell wall lipids induce a NKT cell response. A preceding study from this group identified Phosphatidylinosotol-mannoside (PIM) as lipid antigen, presented by CD1d and recognized by NKT cells (Fischer et al., 2004). The CD1d binding PIM preparation also induced a potent IFNγ release by splenic Vα14Jα281 tg T cells in a CD1d-dependent manner (Fischer et al., 2004)and Figure 21). In contrast to αGalCer, PIM did not induce IL-4.

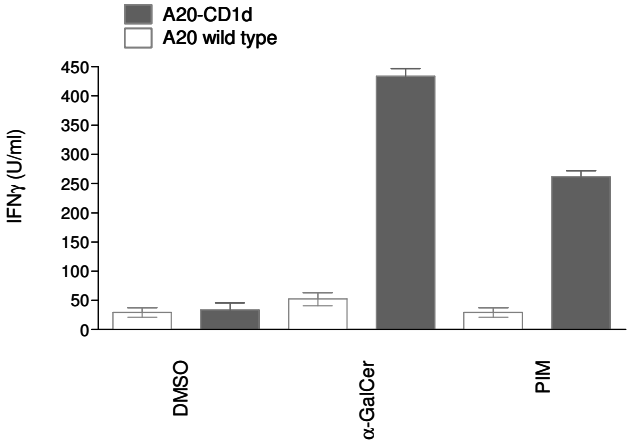


Figure **21**: Recognition of mycobacterial PIM by CD1d-restricted T cells. A20-CD1d and A20 APC were loaded with lipids, splenic T cells from Vα14Jα281 tg mice were added, and IFNγ was determined 48 h later. Data are expressed as the mean IFNγ concentration, +/- SD of triplicates.

To further analyze PIM-reactive NKT cells, CD1d tetramers were loaded with PIM and FACS staining was performed. Spleen cells of C57BL/6 mice were isolated and stained with CD1d tetramers loaded with αGalCer or PIM. Tetramer staining of C57BL/6 splenocytes revealed 2.2% αGalCer-CD1d tetramer-positive and 0.6% PIM-CD1d tetramer-positive NKT cells (Figure 22).

Moreover, CD1d-tetramer staining revealed that in C57BL/6 mice ~ 30% of liver NKT cells reacted to

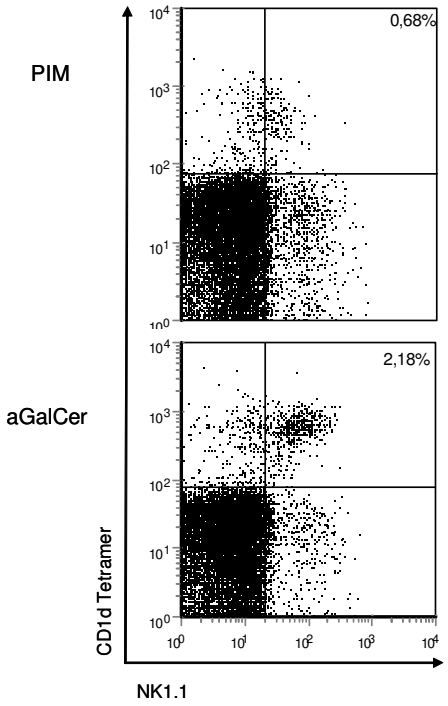


Figure **22**: Tetramer staining of spleen NKT cells from C57BL/6 mice. CD1d-tetramers were loaded with αGalCer or Phosphatidylinositol-mannoside (PIM). Cells were stained with APC-conjugated anti-CD3, FITC-conjugated anti-NK1.1 and PE-conjugated anti-TLR2. Cells shown were gated for CD3 expression. Percentages of positive cells are indicated in the quadrants and are shown in European format. Dead cells were excluded by PI-staining.

αGalCer-CD1d tetramers and ~ 0.26% to PIM-CD1d tetramers (Figure 23).

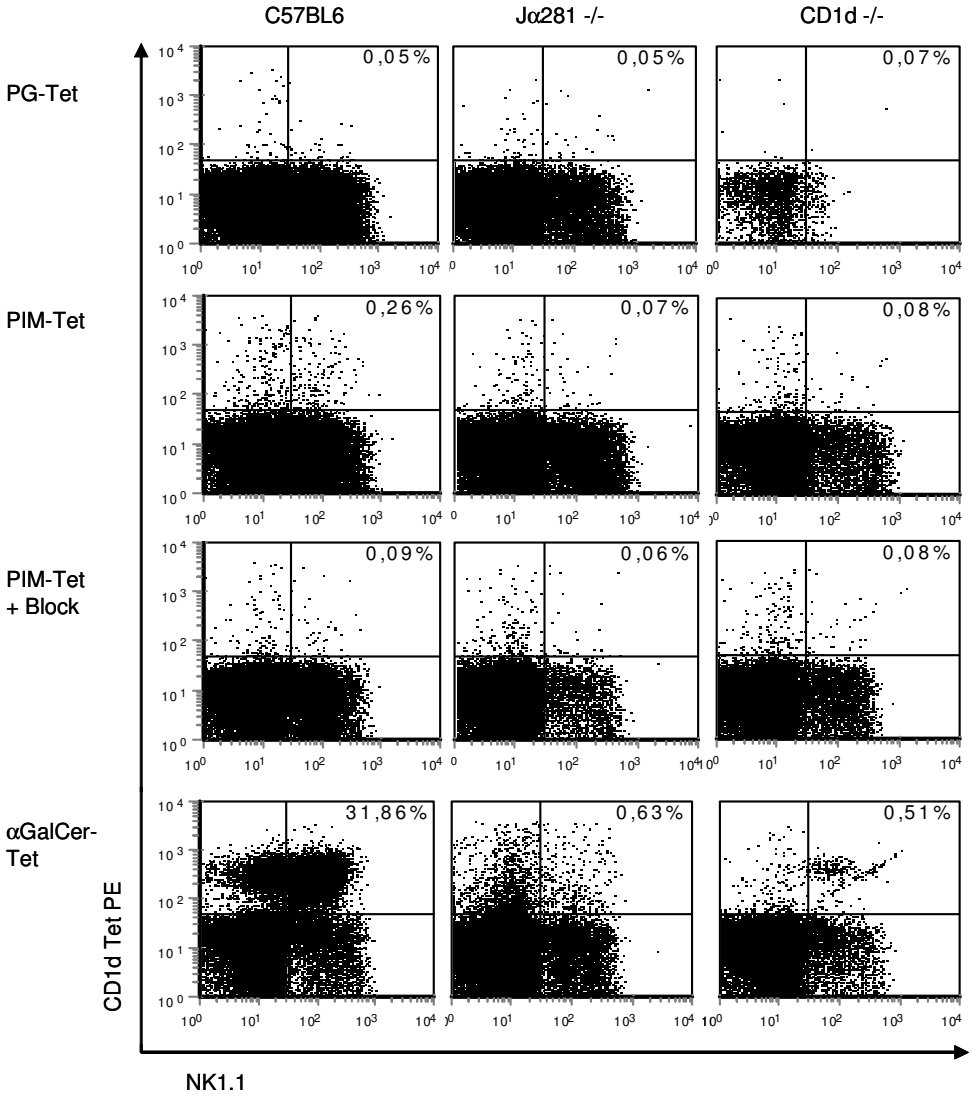


Figure **23**: Tetramer staining of liver lymphocytes from C57BL/6, CD1d -/-, or Jα281-/-mice. Cells were stained with APC-conjugated anti-CD3 mAb, FITC-conjugated anti-NK1.1, and PE-conjugated CD1d tetramers loaded with the indicated lipids. Unlabeled PIM-CD1d tetramers were used for blocking. Dead cells were excluded by PI-staining. Percentages of positive cells are indicated in the quadrants and are shown in European format. Block: unlabeled PIM-CD1d-tetramer.

The tetramer-TCR interactions were blocked by preincubation of cells with unlabeled PIM-CD1d tetramers, confirming the specificity of the tetramer construct. Tetramers loaded with PG, a lipid shown to bind CD1d but not to induce a T cell response, did not stain liver T cells from B6 mice (Fischer et al., 2004). In $CD1d^{-/-}$ and $J\alpha281^{-/-}$ mice lacking CD1d-restricted T cells, PIM-CD1d tetramer-positive T cells were not detectable, further confirming the specificity of the tetramer staining. These data indicate that ~ 25% of all spleen NKT cells as defined by αGalCer-CD1d tetramer staining, can react to PIM (Figure 23). This result explains why the observed IFNγ response was almost similar to PIM and αGalCer.

αGalCer-reactive NKT cells display a very limited TCR repertoire with constant Vα chain paired with the vβ chains Vβ8, Vβ7, or Vβ2 (Porcelli et al., 1993). To characterize PIM-reactive NKT cells further and determine whether PIM-reactive NKT cells could be a subset of αGalCer-reactive NKT cells, liver NKT cells of Vα14Jα281 were stained with antibodies against a variety of Vβ chains and with PIM- or αGalCer- loaded CD1d tetramers. Forty-eight % of αGalCer- and 0.56 % of PIM-reactive NKT cells were double positive for CD1d tetramer and Vβ8 staining (Figure 24) and 14 % of αGalCer- and 0.2 % of PIM-reactive NKT cells were detected for Vβ7 expression. Vβ2 and CD1d tetramer double-positive NKT cells were not detectable for either αGalCer or PIM-reactive NKT cells. Furthermore, only 0.36 % αGalCer- and 0.04% of PIM-reactive NKT cells were double positive for CD1d tetramer and Vβ11 (Figure 24). These results demonstrate predominant expression of Vβ8 and Vβ7, revealing no differences in Vβ usage between αGalCer and PIM-reactive NKT cells.

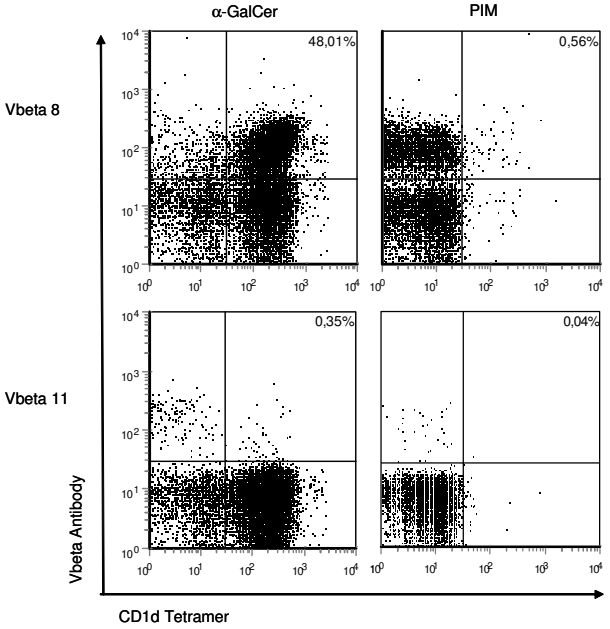


Figure **24**: Vβ expression of liver NKT cells from Vα14 tg mice. Cells were stained with APC-conjugated anti-CD3 mAb, FITC-conjugated anti-Vβ chains, and PE-conjugated CD1d tetramers loaded with the indicated lipids. Dead cells were excluded by PI-staining. Percentages of positive cells are indicated in the quadrants and are shown in European format.

To investigate for differences between PIM and αGalCer-reactive NKT cells during an infection, C57BL/6 mice were infected with $2x10^6$ *M. bovis* BCG. Mycobacteria were injected i.p. into 3 mice per group and after 1, 3, 8 and 270 days (d), spleen and liver were removed and analyzed for frequencies of NKT cells. NKT cells were stained by anti-NK1.1 antibody and CD1d tetramer. The αGalCer-CD1d tetramer$^+$ and NK1.1$^+$ liver NKT cells showed an increase in cell numbers 1 d after infection, but a significant decrease in cell numbers was detected on d 3 and d 8 after infection. These cells started to reappear 270 d after infection, indicated by slightly increased cell numbers. The αGalCer-CD1d tetramer$^+$ but NK1.1$^-$ cells showed a slight increase in cell numbers during d 1 to d 8 after infection and dropped back to initial cell numbers after 9 months (Figure 25).

Relative results from FACS analysis of Vβ usage and analysis of NKT cell numbers during mycobacterial infections show no major differences between αGalCer-reactive and PIM-reactive NKT cells. These results suggest that PIM specific NKT cells constitute a smaller subset within the NKT cell population as defined by αGalCer recognition.

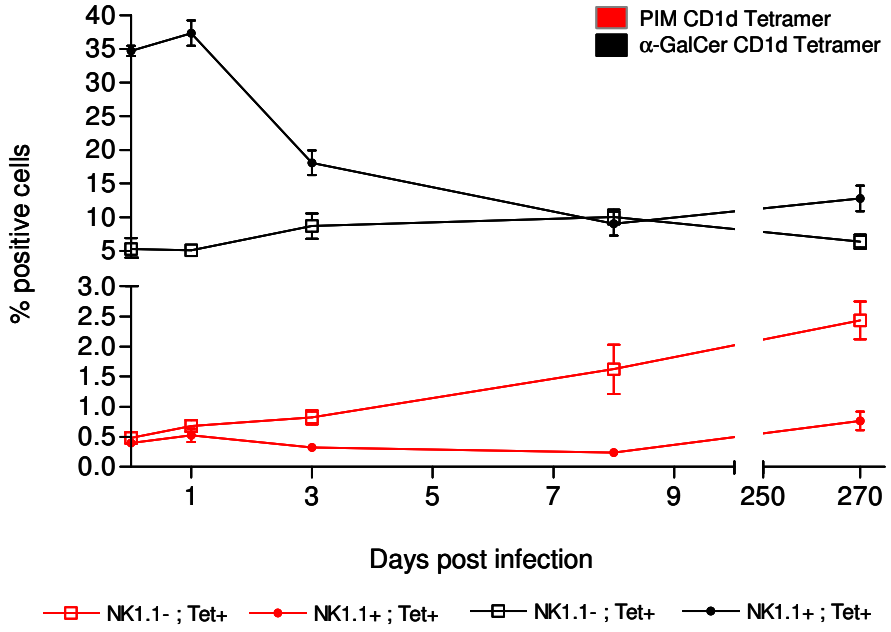


Figure **25**: NKT cell numbers during infection with *M. bovis* BCG. C57BL/6 mice were infected i.p. with $2x10^6$ bacteria. Liver lymphocytes were stained with APC-conjugated anti-CD3 mAb, FITC-conjugated anti-NK1.1, and PE-conjugated CD1d tetramers loaded with the indicated lipids. Dead cells were excluded by PI-staining. Data are expressed as mean percentage of positively stained cells, +/- SD of 3 mice. Infection studies were done twice

5.4 TCR vs. TLR induced NKT cell activation

NKT cell activation can be mediated directly by CD1d presented antigens recognized by the NKT cell TCR (Kawano et al., 1997). Alternatively, recognition of conserved bacterial structures through Toll-like receptors on APCs activates the APC to produce IL-12. This IL-12, in turn, activates NKT cells which recognize their endogenous lipid antigen presented by CD1d (Brigl et al., 2003).
As described in the previous paragraph, we identified PIM as a CD1d presented, natural NKT cell antigen. The next experiments were designed to follow up on this project and to investigate further antigenic capacities of mycobacterial lipids.

5.4.1 Mycobacterial PIMs induce NKT cell activation via TCR and TLR

To further asses the role of TLRs in activation of NKT cells and to discriminate direct, TCR mediated and indirect, TLR mediated NKT cell activation, PIM containing lipid fractions were isolated from *M. bovis* BCG. PIM containing lipid fractions were isolated as described in section 4.2.4. Crude lipid extracts were analyzed for their capacity to activate human cells through TLR2. Antigens examined were: crude PIM extracts, whole lipid extracts, phosphatidylinositol (PI) and phospolipase A treated PIM (lyso-PIM). PI binds to CD1d but fails to elicit a CD1d-mediated NKT cell response, Lyso-PIM, lacking one acyl-chain is devoid of the ability to activate NKT cells via CD1d presentation (Fischer et al., 2004). Bacterial lipopeptide (BLP; Pam_3CSK_4) was shown to activate a TLR2 transfected cell line (Aliprantis et al., 1999a). BLP was used as positive control. TLR2 activation was detected as described in section 4.1.14. The 293-cells transfected with exogenous hTLR2 activate NF-κB in response to BLP. In order to asses NF-κB activation, an NF-κB luciferase reporter gene consisting of an artificial promotor composed of a multimer of 5 NF-κB sites driving the firefly luciferase gene, is cotransfected with a constitutively active Renilla-luciferase reporter gene. Antigens were dissolved in DMSO, incubated with TRL2 expressing 293-cells for 8 h and subsequently NF-κB activity was measured. The 293-cells were transfected with the empty vector (pRKN) to detect TLR2-independent NF-κB activation. Lipids were used at a range of 0.02 - 5 µg/ml.
Whole lipid extracts as well as crude PIM extracts were recognized by human TLR2 and induced significant NF-κB induction in a dose dependent manner. PI did not induce NF-κB but interestingly, lyso-PIM did neither (Figure 26). Moreover, αGalCer was not recognized by TLR2 and failed to activate NF-κB (data not shown). Unspecific NF-κB activation was minimal and substracted from depicted values. A fold induction of ≥ 4 is considered as significant TLR2-mediated NF-κB induction.

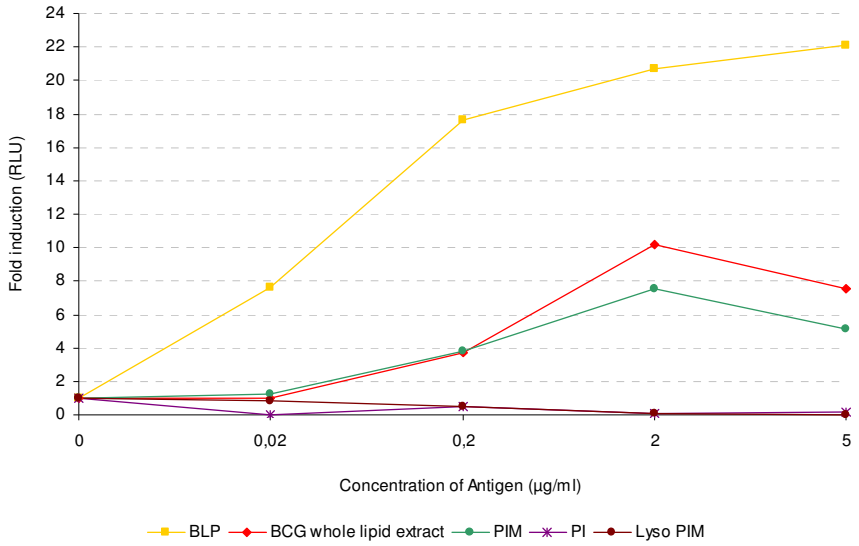


Figure **26**: NF-κB activation triggered through TLR2-mediated recognition of lipids isolated from *M. bovis* BCG. The 293 cells transfected with human TLR2 were incubated with different lipids and lipid extracts for 8 h at the concentrations indicated. NF-kB induction through hTLR2 was detected using a firefly dual luciferase driven reporter assay. BLP: Bacterial lipopeptide (Pam_3CSK_4).PIM: phosphatidylinositolmannoside; PI: phospatidylinositol; lyso PIM: phospholipase A treated PIM. Data are expressed as the mean fold induction of NF-κB, +/- SD of triplicates. RLU: relative light units.

In comparison to PIM, PI lacks the mannose sugar part and phospholipase A treatment of PIM results in the removal of one acyl chain (Figure 27; PIM shown as PIM_4).

This result suggests that both the structure of the lipid part and the presence of a certain sugar moiety is important for TLR2 recognition and subsequent NF-κB activation.

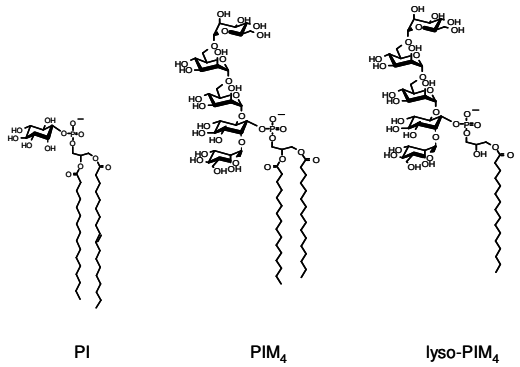


Figure **27**: Structures of phospatidylinositol (PI), phospatidylinositol-tetramannoside (PIM_4) and lyso-PIM_4.

Crude lipid fractions were further fractionated and purified by liquid chromatography employing a silica gel column and increasing mixtures of chloroform / methanol. After isolation, lipid fractions were analyzed by one-dimensional HPTLC (Figure 28).

Fractions were dissolved in a mixture of chloroform / methanol and separated on HPTLC using a mixture of chloroform/acetic acid/methanol/water in a 40/25/3/6 relation, respectively. Crude PIM extracts and pure $PIM_{1,2}$ were used as markers and chromatography buffer was included to discriminate positive staining from buffer contamination. Lipids were detected by α-naphtol. Using α-naphtol, carbohydrate residues are stained blue, whereas red staining indicates absence of carbohydrates. Figure 28 depicts lipid fractions separated by HPTLC showing different lipids within isolated fractions. Fractions 1 - 13 contain lipids that were more soluble in the HPTLC

solvent and characteristic bands for phosphatidylinositol-mono/di-mannoside ($PIM_{1,2}$) was detected. Higher mannosylated PIMs, like Phospatidylinositol-tetramannoside (PIM_4), was detected within fractions 14 - 23 indicated by the dense spot at the base each lane. Remaining buffer ingredients appear to be present in fraction 5.

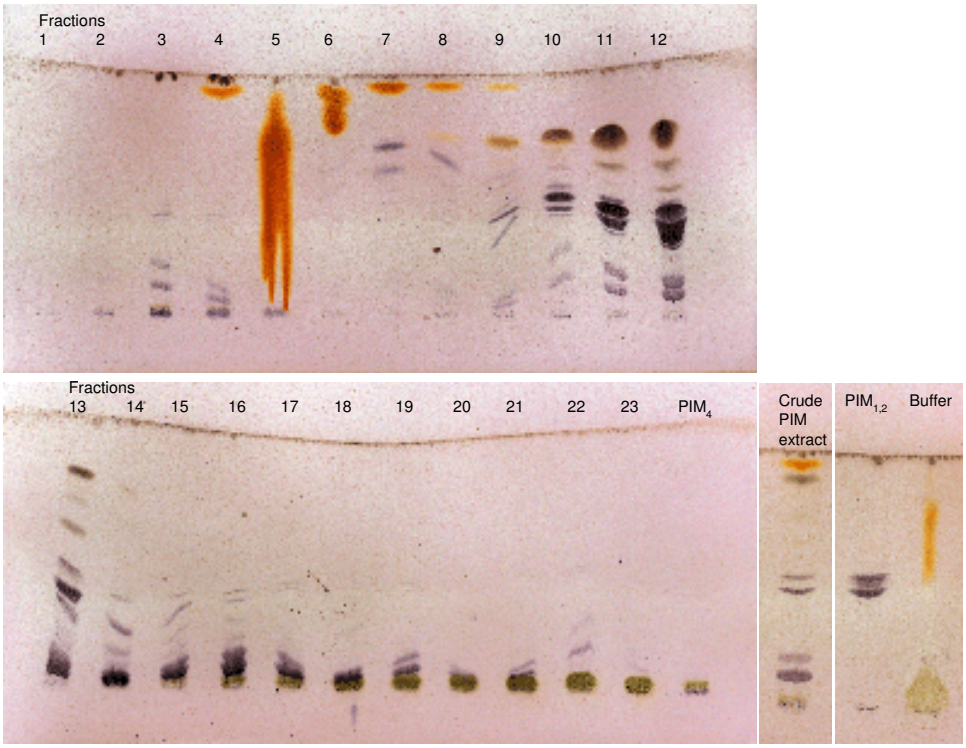


Figure **28**: Thin layer chromatography of lipid fractions isolated from *M. bovis* BCG. Lipids were fractionated using a silica column and eluted by a chloroform / methanol gradient. Subsequently, lipids were separated in one dimension using HPTLC plates and detected by α-naphtol staining.PIM_4, phosphatidylinositol-tetra-mannoside; $PIM_{1,2}$ phosphatidylinositol-mono /di-mannoside; buffer: Sepharose 200 column chromatography buffer

To analyze the isolated lipid fractions for their potential to elicit a CD1d-dependent NKT cell response, $3x10^4$ HeLa cells were incubated overnight with different lipid fractions at 1 µg/ml. On the next day, $1.5x10^5$ human NKT cells from a polyclonal NKT cell line were added. After 6 h, TNFα was measured in the cell culture supernatant by ELISA. αGalCer was used as positive control and DMSO as negative control since lipids were dissolved in DMSO. αGalCer and fraction 7 elicited a potent NKT cell response detected by TNFα production. None of the other lipid fractions provoked a NKT cell response (Figure 29).

The same fractions were also analyzed for their capacity to be recognized by human TLR2 and thereby inducing NF-κB activation. Lipids were dissolved in DMSO and used at a concentration range from 1 - 5 µg/ml. TLR2-mediated NF-κB activation was measured the same way as described above. *M. bovis* BCG whole lipid extracts and *M. tuberculosis* lysate were also examined. BLP was used at a concentration of 0.1 - 100 ng/ml as positive control.

Transfectants containing the empty vector lacking TLR2 were added as negative control for TLR2 independent NF-κB activation. Lipid fractions 7, 16, 19 and 21 induced NF-κB activation up to a concentration of 4 µg/ml and showed a decline of induction with higher concentrations. *M. bovis* BCG whole lipid extracts and *M. tuberculosis* lysate were recognized by human TLR2 and induced NF-κB increasingly strong with increasing concentrations. Lipid fractions 11 and 12 were not capable of inducing a response. Unspecific NF-κB activation was minimal and subtracted from depicted values (Figure 30). A fold induction of ≥ 4 is considered as significant TLR2-mediated NF-κB induction.

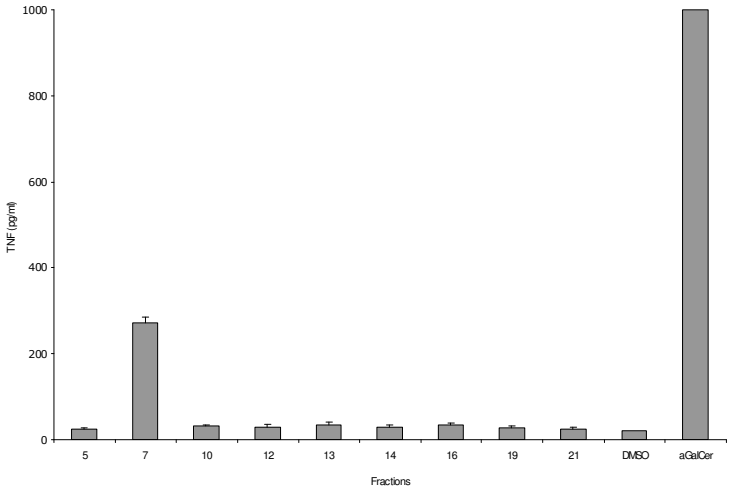


Figure **29**: TNFα release by human NKT cells. CD1d expressing HeLa cells ($3x10^4$) were incubated overnight with different lipid fractions. NKT cells ($1,5x10^4$) were added for 6h and TNFα was measured in the cell culture supernatant. Results shown are in triplicates.

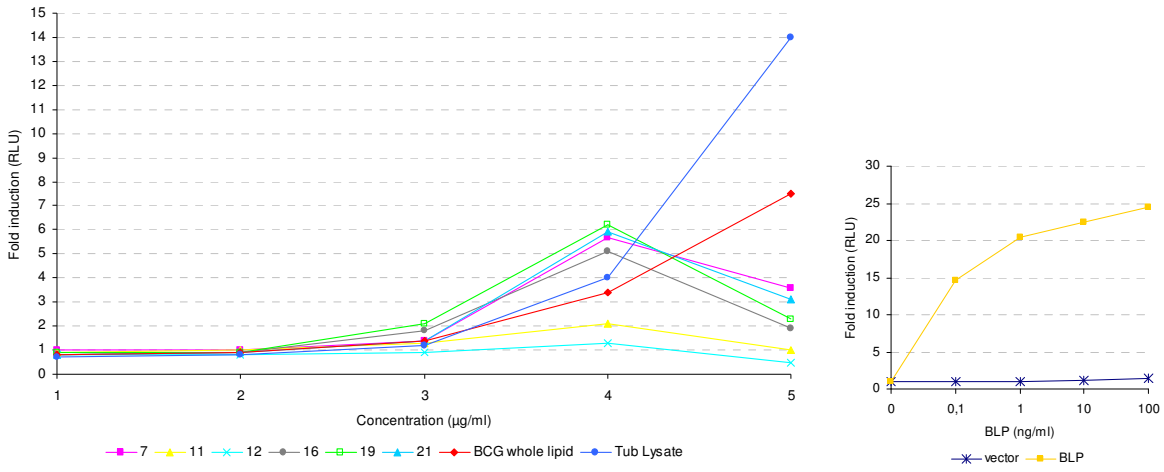


Figure **30**: NF-κB activation triggered through TLR2-mediated recognition of lipids isolated from *M. bovis* BCG. (Left) Crude PIM fractions were further fractionated and purified and the resulting fractions were analysed for their capacity to induce TLR2 activation. (Right) BLP or vector control. The 293 cells transfected with human TLR2 were incubated with different lipids and lipid extracts for 8 h at the concentrations indicated. NF-kB induction through hTLR2 was detected using a firefly dual luciferase driven reporter assay. BLP: Bacterial lipopeptide (Pam3CSK4). Data are expressed as the mean fold induction of NF-κB, +/- SD of triplicates

Experiments for CD1d-mediated NKT cell activation and TLR2-mediated NF-κB activation revealed that lipid fraction 7 induced responses in both assays. Lipid fractions 16, 19 and 21 induced only TLR2-mediated responses. Therefore, fraction 7 of the lipid purification seemed to contain a lipid species that has both the capacity to induce human NKT cells specifically and is also recognized by human TLR2. This capacity was absent in fractions 16, 19 and 21.

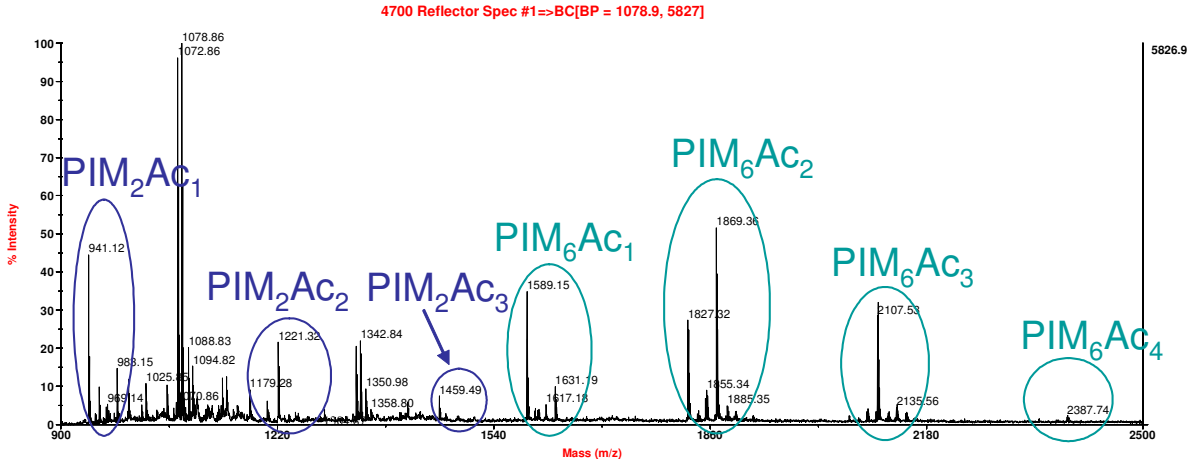
5.4.2 Mass-spectrometry analysis of TLR and TCR activating lipid fractions

As a consequence, MALDI-MS was performed to reveal structural differences within lipid fractions 7, 16, 19 and 21. The aim was to detect the NKT cell activating lipid in fraction 7. Moreover, analysis of lipid structures would reveal structural elements required for TLR2 recognition. Lipids were dissolved in a mixture of chloroform / methanol at a concentration of 5 µg/ml and mixed with 2,5-Dihydroxybenzoic acid (DHB) as matrix for MALDI-MS. The spectra were recorded in both positive and negative mode and at two types of expansion: m/z 500 – 3,000 and m/z 1,000 – 3,000. Internal calibration was achieved by using synthetic peptides with known molecular mass as internal markers. Negative mode MALDI-MS did not yield clear spectra but using positive mode parameters, MALDI-MS was successful. The reference spectrum of crude PIM extracts was recorded by Martine Gilleron and Germain Puzo at the Institut de Pharmacologie et de Biologie Structurale, Centre National de la Recherche Scientifique, Toulouse Cedex, France (Figure 31). MALDI-MS spectra of lipid fractions 7, 16, 19 and 21 were recorded at the mass-spectrometry facility of the MPI for Infection Biology.

In fraction 7, a cluster of smaller peaks at the range of 1233.97 to 1640.61 Da was detected without any predominant peak within this cluster. Some of the peaks could be attributed to known PIM structures: PIM_2Ac_1 (Mass 941.72) and PIM_6Ac_1 (Mass: 1598.37) were clearly detected. A mass of 1456.49 was detected which is close to PIM_2Ac_3 which has a mass of 1459.49. Peaks with masses higher than 1640.61 could not be identified and other peaks within the detected cluster could not be assigned. Lower case letters indicate numbers of mannoses or acylation grade, e.g. PIM_6Ac_1 is monoacylated phosphatidylinositol-hexamannoside. A more definite result was obtained with fractions 16, 19 and 21. PIM_6Ac_2 with two C_{18} acyl chains (1869.61 Da) or two C_{16} acyl chains (1828.7 Da), PIM_6Ac_3 (2108.15 Da) and PIM_6Ac_4 (2388.42 Da) were detected in fractions 16, 19 and 21. PIM_2Ac_3 (1495.96) was detected in fractions 16 and 21 and PIM_2Ac_2 (1217.98) was detected in fraction 21. Minor peaks between the molecular masses of 995.51 to 1525.06 were detectable in fractions 16, 19 and 21 with a cluster of them appearing in fraction 21 (Figure 31). Due to the lack of clear peaks in fraction 7 no individual compound could be pinpointed with the ability to

activate NKT cells as well as being recognized by TLR2. Peaks, indicating the masses of highly mannosylated PIMs were not detected in fraction 7 but present in all other fractions, indicating TLR2 recognition of highly mannosylated PIMs but no recognition of lower mannosylated PIM species.

a) Standard MALDI-MS spectrum of a crude PIM purification



b) Fraction 7

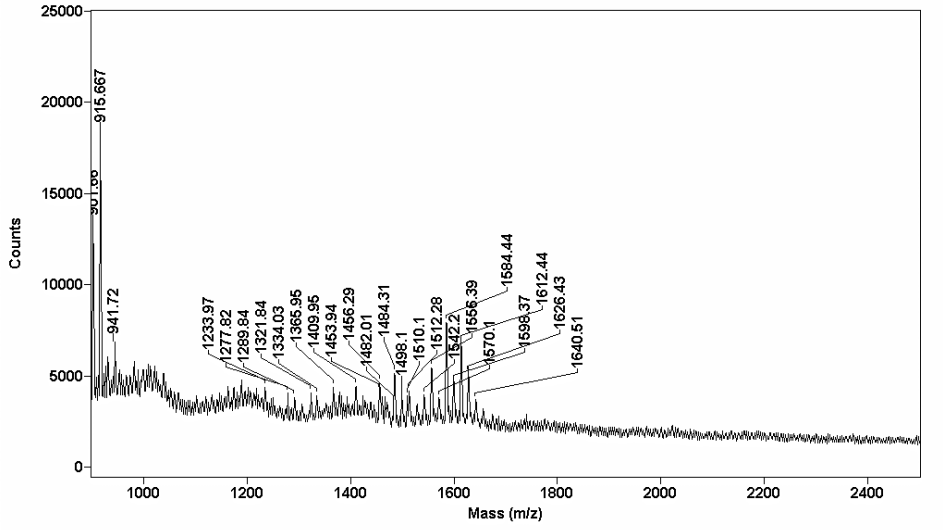
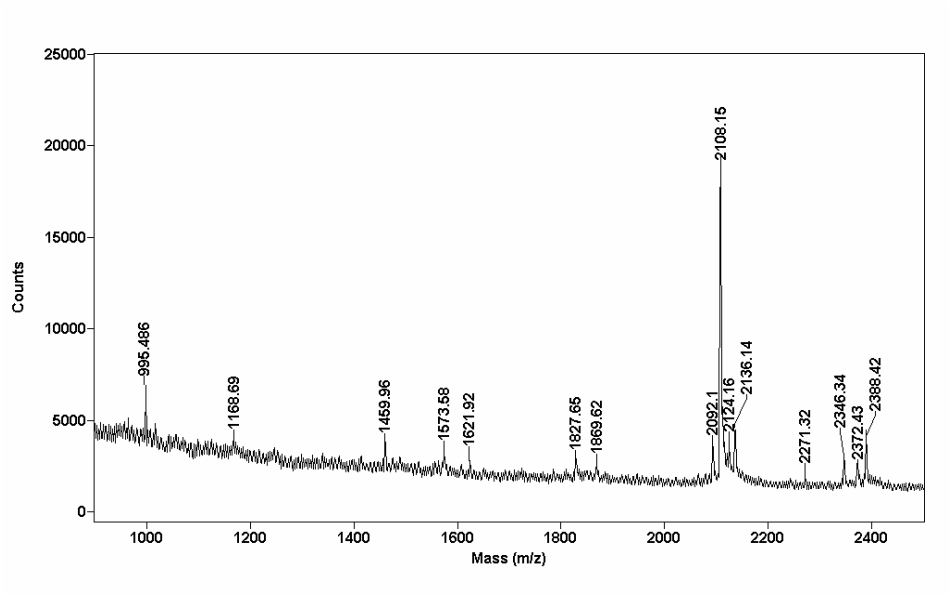


Figure **31**: MALDI-MS analysis of lipid fractions isolated from *M. bovis* BCG. Lipids were dissolved in a mixture of $CHCL_3$-CH_3OH at a concentration of 1 mg/ml. DHB was used as matrix and spectra were recorded in positive mode at an expansion of m/z 1000 3000. a) Reference spectrum of crude PIM extracts showing the respective lipids for each encircled peak. b) lipid fraction 7.

c) Fraction 16



d) Fraction 19

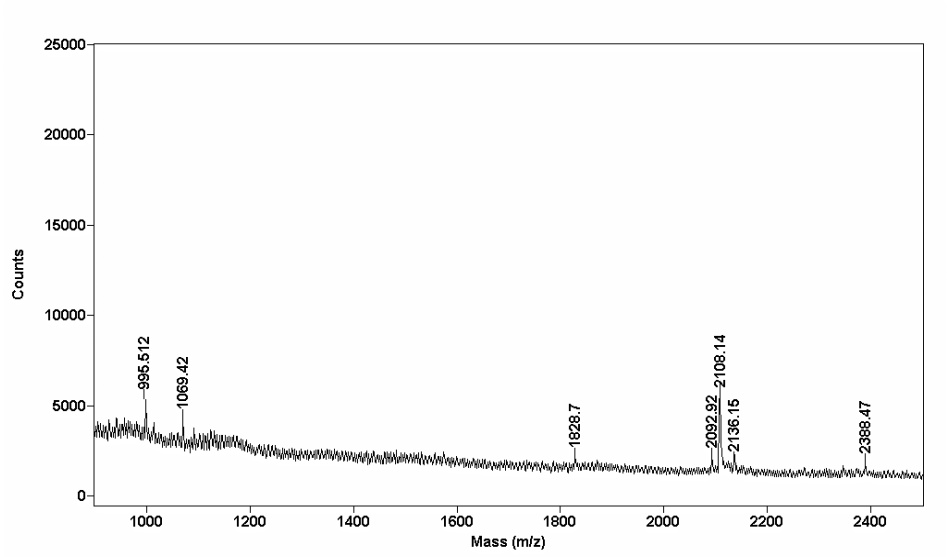


Figure **31** continued: MALDI-MS analysis of lipid fractions isolated from *M. bovis* BCG. Lipids were dissolved in a mixture of $CHCL_3$-CH_3OH at a concentration of 1 mg/ml. DHB was used as matrix and spectra were recorded in positive mode at an expansion of m/z 1000 3000. c) lipid fraction 16 d) lipid fraction 19.

e) Fraction 21

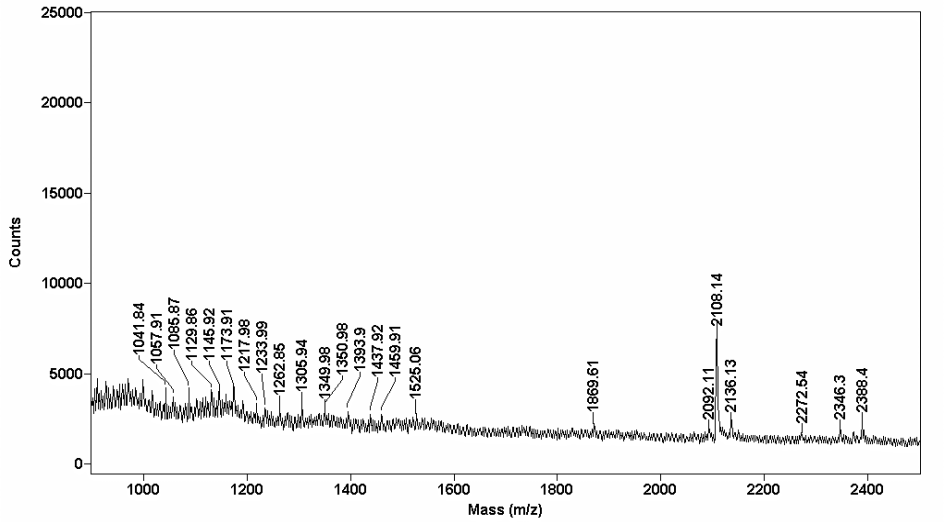


Figure **31** continued: MALDI-MS analysis of lipid fraction isolated from *M. bovis* BCG. Lipids were dissolved in a mixture of $CHCL_3$-CH_3OH at a concentration of 1 mg/ml. DHB was used as matrix and spectra were recorded in positive mode at an expansion of m/z 1000 3000. e) lipid fraction 21

5.4.3 Toll-like receptor expression by NKT cells

TLR2 and TLR4 recognize a wide range of bacterial cell wall products including lipoproteins, LAM, LPS and peptidoglycan and contribute to innate immune responses to mycobacterial infections (Medzhitov, 2001; Underhill and Ozinsky, 2002). To examine if NKT cells could recognize bacterial antigens via TLRs, FACS analysis of extracellular and intracellular expression of TLR2 and TLR4 on NKT cells was performed.

Spleen and liver NKT cells of Vα14Jα281 tg mice were co-stained with antibodies against murine TLR2 or TLR4 as well as NK1.1 and CD3 (Figure 32). Surface expression of TLR2 on NKT cells could not be detected above background level stained by an IgG2b isotype control antibody. Analysis of TLR4 expression on NKT cells was performed using cells from Vα14Jα18 tg as well as TLR4$^{-/-}$ mice. Furthermore, TLR4 blocking antibodies were employed. TLR4 expression was detected for 0.12 % and 0.17 % of spleen and liver NKT, respectively. This positive staining was decreased to 0.04 % and 0.1 % positive cells in spleen and liver, respectively by the use of unconjugated anti-TLR4 blocking antibodies. TLR4$^{-/-}$ mice showed TLR4 expression on 0.02 % spleen and 0.07 % liver NKT cells. This indicates unspecific antibody binding.

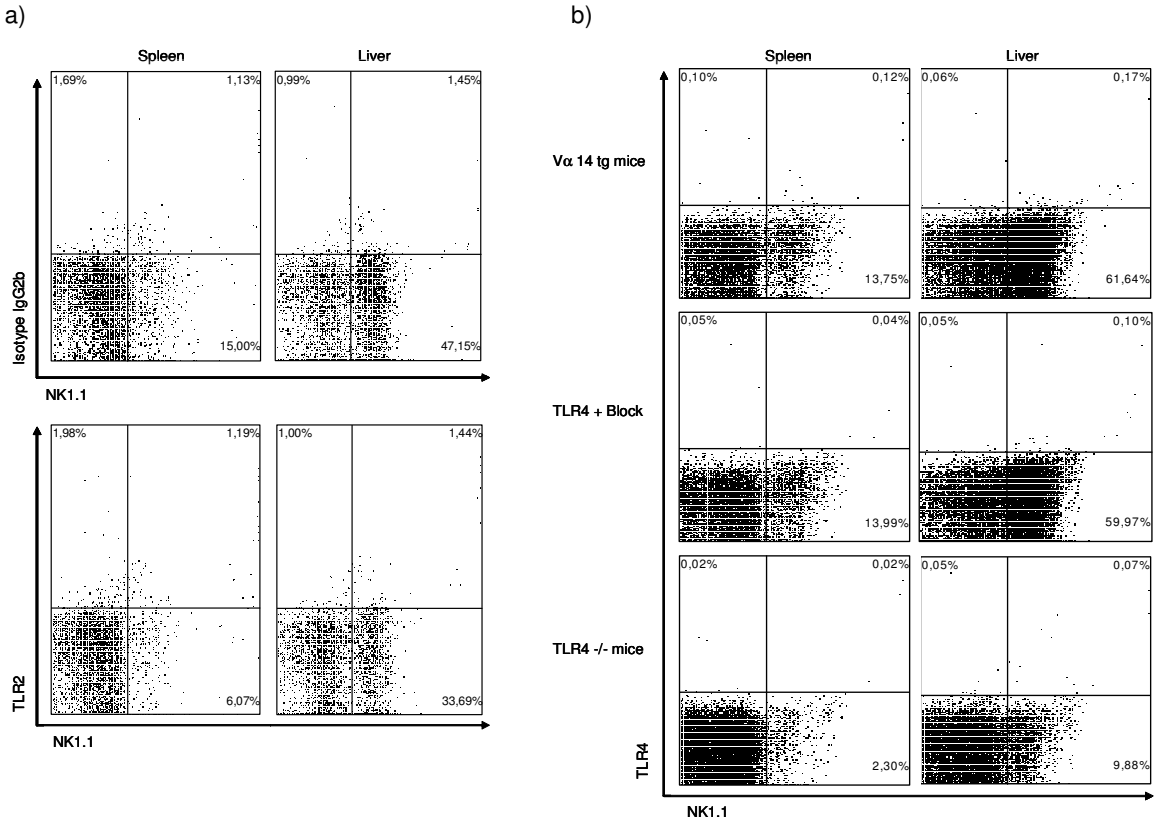


Figure **32**: a) TLR2 expression of murine NKT cells: Spleen and liver NKT cells of Vα14 transgenic mice were stained for TLR2 expression. Cells were stained with APC-conjugated anti-CD3, FITC-conjugated anti-NK1.1 and PE-conjugated anti-TLR2. Cells shown were gated for CD3 expression. IgG2b antibodies were used as isotype control staining. One of three experiments is shown. b) TLR4 expression of murine NKT cells. Spleen and liver NKT cells of Vα14 transgenic mice and TLR4 knock out (-/-) mice were used. Cells were stained with APC-conjugated anti-CD3, FITC-conjugated anti-NK1.1and PE-conjugated anti-TLR4. Block: unconjugated anti-TLR4 antibody. Cells shown were gated for CD3 expression and percentages of positive cells are indicated in the quadrants and are shown in European format.

However, the detection of minute percentages of TLR4 positive NKT cells cannot be considered as a TLR4$^+$ NKT cell population. The low expression level was predominantly unspecific not depicting a clearly defined population of TLR4$^+$ NKT cells by FACS. FACS analysis of intracellular TLR4 expression did also not reveal any TLR4$^+$ NKT cells.

6 Discussion

NKT cells possess very diverse capacities and are involved in a variety of immune responses (Godfrey and Kronenberg, 2004).
A unique gene expression pattern of NKT cells was depicted by transcriptome analysis. The pattern showed an activated, effector phenotype, expression of distinct cytokine- and chemokine receptors and a tight control of autoreactivity. Additionally, discovery of shared gene expression patterns with other, distinct lymphocyte populations revealed innate and adaptive immunity-like features of NKT cells. Analysis of activated NKT cells showed substantial increase in gene expression for effector and immuno-regulatory factors, such as cytokines and chemokines combined with strict regulation of NKT cell activity and apoptosis.

Analysis of NKT cell stimulation by the endogenous antigen iGb3 under normal physiological conditions and in lysosomal storage disorders revealed that regulation of antigen availability is crucial for NKT cell activation and control of NKT cell activity.

In addition, mycobacterial antigens for NKT cells were identified and NKT cells, reactive to these lipids, were characterized. Furthermore it was shown that distinct mycobacterial lipids are capable of eliciting both, a specific CD1d-mediated NKT cell activation and a broad immune activation by TLR2 activation.

6.1 Defining NKT cells through their gene expression pattern

One aim of this thesis was to define NKT cell properties by analysis of their gene expression pattern. Global analysis of genes, differently regulated in NKT cells in comparison to other lymphocyte populations provides a view on the diverse functions and properties of NKT cells. Up to date, NKT cell characterization by means of gene expression patterns has not been reported.

Specific attribution of genes to distinct cell populations by microarray analysis requires tight control of sample preparation. It is indispensable that genes, detected in microarray analysis are truly cell type specific genes. This was achieved by purification of cells via MACS and FACS sorting, exclusion of dead cells and maintenance of the cells at 4°C. This ensured that isolated population were highly pure, viable and not activated. Activation of cells would result in alterations in gene expression during the purification.

The lower purity of NKT cell sorts was surprising but can be attributed to two factors. Limitations of the FACS sorter, for instance to exclude all dead cell and cell doubles, and quantitative variation of NK1.1 and Vα14 TCR surface expression by NKT cells (Emoto et al., 1999b; Hammond et al., 1999). Analysis of sorted NKT cells detected a well-defined cell population with very little contamination (Figure 7). Specificity of microarray analysis was additionally controlled by combining results from two independent experiments. Only expression of genes found up- or downregulated in both experiments were considered cell type specific. Additionally, only genes with a minimum of 2 fold differences in expression levels and P-values smaller than 0.05 were chosen.
Methods employed are the most sophisticated tools available at present and controls chosen were designed to ensure reliability of microarray results. A significant number of genes already known to be expressed by NKT cells were detected in microarray analysis, thus validating the fidelity and specificity of microarray derived data.

6.1.1 The NKT cell: portrayal by means of gene expression profiling

6.1.1.1 A unique genetic imprint of NKT cells

Genes with a higher expression in NKT cells compared to other lymphocyte populations portray a set of genes exclusively expressed in NKT cells or at least exhibiting a unique expression pattern. Among these 15 genes, expression of only 3 genes was previously described for NKT cells (Table 2). These are: CXCR3, IL-12 receptor-β1 and integrin-α4β1 (Franitza et al., 2004; Kim et al., 2002; Kitamura et al., 1999).

Rapid NKT cell responses are due to expression of chemokine and cytokine receptors

CXCR3 is a chemokine receptor for homing to non-lymphoid tissue. IL-12 receptor binds the pro-inflammatory cytokine IL-12 and integrin-α4β1 is involved in the recruitment of T cells to sites of inflammation. In addition to CXCR3 and IL-12 receptor-β1, NKT cells expressed CCR3 and IL-17 receptor-B. CCR3 is also involved in attraction of lymphocytes to sites of inflammation and IL-17 receptor-B binds IL-17, a pro-inflammatory cytokine like IL-12. IL-12 receptor is not expressed on resting, conventional T cells and IL-17 induces production of other cytokines, e.g. IL-6 and IL-8. The strong ability of NKT cells to rapidly respond to pro-inflammatory cytokines can be explained by the expression of IL-12 receptor-β1 and IL-17 receptor-B on resting, non-activated NKT cells. Additionally, elevated expression of chemokine receptors CXCR3 and CCR3 implies rapid recruitment to sites of inflammation and fast response to inflammatory mediators. This set of chemokine/cytokine receptors enable NKT cells to rapidly respond to external stimuli.

NKT cells display an activated, effector phenotype

The recruitment of leukocytes into tissues depends on a series of adhesive and activation steps mediated by adhesion molecules and chemokine–receptor interactions (Johnston and Butcher, 2002). Expression of chemokine receptors and adhesion molecules on T cells is developmentally regulated, while chemokine expression is often tightly controlled by cytokines that restrict their tissue distribution. This results in distinct migratory patterns of leukocyte subsets (Kunkel and Butcher, 2002). For example, all naïve T cells uniformly express L-selectin and CCR7, which mediate access to lymphoid tissues through high endothelial venules and have a role in T-cell localization in the paracortex of lymph nodes. By contrast, memory and effector T cells are heterogeneous in their expression of chemokine receptors and adhesion molecules, and have diverse trafficking behaviors (Kim et al., 2002).
The chemokine receptor and adhesion molecule expression pattern of NKT cells resemble that of activated, effector T cells, i.e. expression of chemokine receptors for homing to non-lymphoid tissue (Gumperz et al., 2002; Lee et al., 2002) and Table 2). Decreased expression of L-selectin and vimentin indicates reduced trafficking to lymph nodes and acquired ability for extravasation. Increased expression of MMP-9 contributes to the activated phenotype of NKT cells since it is important in tissue remodeling and extravasation. Moreover, Emoto et al described the expression of LFA-1 on NKT cells and demonstrated that LFA-$1^{-/-}$ mice exhibit markedly decreased numbers of NKT cells in the liver, stressing the importance of tissue homing receptors for NKT cell localization (Emoto et al., 1999a). LFA-1 belongs to the family of β_2 integrins and is involved in initiating leukocyte transmigration and extravasation. In contrast, decreased expression of integrin-$\alpha 4\beta 1$ by NKT cells implies reduced trafficking to sites of inflammation. This discrepancy cannot be explained. However, NKT cells contain distinct subsets with differential expression of chemokines, chemokine receptors and adhesion receptors. This could provide a mechanism to deliver specific NKT cell subsets to distinct tissues and deliver a specific immuno-regulatory function.

Proliferation of resting NKT cell is tightly controlled

BLK, caspase-1, caspase-11 and KLF2 are involved in regulation of apoptosis. Whereas BLK is pro-apoptotic and upregulated in NKT cells, caspase-1 and caspase-11 are not only involved in regulation of apoptosis. Caspase-1 is involved in cytokine processing (Pirhonen et al., 1999) and caspase-11 is a mediator of septic shock responses and induced in most cells upon pro-inflammatory stimuli. Activation of caspase-1 is under the control of caspase-11. Expression of caspase-11 is induced by LPS and caspase-$11^{-/-}$ mice exhibit significantly reduced levels of IL-1α and IL-1β after LPS stimulation (Wang et al., 1996). IL-1 in turn, is involved in septic shock (Kang et al., 2002). Additionally, IL-1β inhibits the induction of KLF2 (SenBanerjee et al., 2004). KLF2, equipped with anti-apoptotic capacities, was

downregulated in NKT cells. In contrast to BLK, caspase-1, caspase-11 and KLF2 exhibited decreased expression levels in NKT cells (Table 2).
Increased expression of BLK together with downregulation of caspase-1, caspase-11 and KLF-2 demonstrate that immunoregulatory functions and proliferation of NKT cells are specifically controlled. Naïve, resting NKT cells were described to be autoreactive even in the absence of antigen stimulation. Upregulation of pro-apoptotic genes and downregulation of anti-apoptotic genes in non-activated NKT cells ensures strict control of NKT cell activity and enables rapid shut down of NKT cell responses. By this mechanism, exacerbated immune reactions leading to pathological consequences can be avoided.

It is apparent that diverse control mechanisms exist to avoid overreaction by NKT cells. This includes upregulation of genes limiting proliferation and selected downregulation of genes with immuno-stimulatory functions. It is noteworthy that IL-17 is involved in amplifying effects of IL-1β as well as in the induction of MMPs (Sylvester et al., 2004). Responses to IL-17 and regulation of IL-1β could therefore be of central importance for NKT cell functions.

6.1.1.2 NKT cells possess innate-immunity like, effector and regulatory functions

Genes with expression levels comparable between NKT cells and either NK cells or conventional T cells characterize NKT cell properties as either properties of innate or adaptive immunity.

Expression of genes coding for lymphotactin, CCL5, CCR2, CXCR4, FasL, LFA-1, IFNγ, NKG2D, CD30 and granzyme B were already described for NKT cells (Emoto et al., 1999a; Faunce and Stein-Streilein, 2002; Gumperz et al., 2002; Kennedy et al., 2000; Kim et al., 2002; Leite-de-Moraes et al., 2000; Matsuda et al., 2002; Metelitsa et al., 2003; Tao et al., 2002) (Table 3). The diversity of these genes demonstrates that NKT cells are able to perform effector functions and act as regulators of immune responses at the interface of innate and adaptive immunity.

Similar expression of lymphotactin, CCR2, CXCR4, FasL, L-CCR, CD30, integrin-α6 and OX40 in NKT cells and NK cells indicate properties of NKT cells related to innate immunity (Table 3). These genes are involved in responses to chemokines and important for cell migration and extravasation to sites of inflammation. Expression levels of IFNγ were comparable in NK and NKT cells and showed decreased expression in conventional T cells. However, production of IFNγ concomitant with IL-4 production is a hallmark of NKT cell as is of central importance in NKT cell function.

Genes with similar expression levels in NKT cells and conventional T cells suggest adaptive immunity-like qualities of NKT cells. Similar expression was detected for CD6, IGTP, IL-4 receptor, IL-7 receptor and interleukin 15. However, IL-4 receptor, IL-7 receptor, interleukin 15 and IGTP are not exclusively expressed in T cells, but stress the ability of NKT cells to respond to broad stimuli like infections and contribute to Th1 and Th2 immune responses (Akashi et al., 1998; Butcher et al., 2005; Carson et al., 1994; Lowenthal et al., 1988).

Additionally, similar expression levels of IL-2 receptor were detected in NKT cells as well as NK cells and conventional T cells. IL-2 promotes proliferation, differentiation, and survival of target cells and augments the innate host defense through its effects on NK cells. Expression of IL-2 receptor on NKT cells underlines their ability to be activated by a wide range of activation signals and participate in various immune responses.

Expression levels of annexin A1, RANTES, CCL6, NKG2D, granzyme B and TLR4 in NKT cells was intermediate between expression levels in NK cells and conventional T cells (Table 3). These genes were upregulated in NKT cells compared to conventional T cells but downregulated to expression levels in NK cells. These genes rather play a role in innate immunity and expression of these genes in NKT cells illustrates properties of innate immunity for NKT cells. However, decreased expression levels in NKT cells compared to NK cells indicates that functions of these genes are not as prevalent as in cells of the innate immunity.

6.1.2 Activated NKT cells: self-control, effector functions and immuno-regulation

Activation of NKT cells is rapid and induces immuno-regulatory and effector function of NKT cells. Concomitant and extensive production of various cytokines is a hallmark of the immuno-regulatory function of NKT cells. In contrast to conventional and regulatory T cells, NKT cells do not only suppress immune responses but actively enhance and modulate reactions to infection and inflammation.

Effector and immuno-regulatory capacities of NKT cells

Microarray analysis of activated NKT cells revealed a substantial increase in expression levels of 10 different cytokine and chemokines (Table 4). Among them, GM-CSF, IFNγ, IL-2, IL-4, IL-13, TNFα, MIP-1α, MIP-1β, lymphotactin were previously reported for αGalCer activated NKT cells (Leite-de-Moraes et al., 2002; Matsuda et al., 2003; Fuss et al., 2004; Harada et al., 2004; Jiang et al., 2005; Kim et al., 2002; Mi et al., 2003). IL-17 production by NKT cells has not been reported. IL-17 is pro-inflammatory and induces the production of

other cytokines. It is noteworthy that expression of some genes, e.g. IL-13 and MIP-1α, detected for activated NKT cells could only be found in distinct NKT cell subsets. However, production of GM-CSF, IL-2, IFNγ, TNFα and IL-4 is characteristic for all activated NKT cells. Concomitant production of Th1 (IFNγ, GM-CSF, IL-2, TNFα) and Th2 (IL-4) cytokines represents a unique capacity of NKT cells. Production of various cytokines and chemokines with diverse regulatory and stimulating properties highlight the capacity of NKT cells to influence and modulate immune responses.

Self-control of NKT cell activity

Strong upregulation of genes controlling cell proliferation was detected for activated NKT cells. Upregulation of the anti-apoptotic gene Gadd45/MyD118 was previously reported in activated NKT cells. Moreover, expression of EGR1, Map_3K_8, c-Myc and Nurr77 was detected in activated NKT cells by microarray analysis (Table 4).

EGR1, c-Myc and Nurr77 are involved in regulation of apoptosis, with EGR1 exhibiting anti-apoptotic properties after cell activation (Adamson and Mercola, 2002; Brunner and Martin, 2004; Mak and Kultz, 2004; Rajpal et al., 2003). Map_3K_8 is part of the Map Kinase pathway transducing growth factor and cytokine-induced activation signals (Beinke et al., 2004). Upregulation of these genes demonstrate induction of apoptosis control in order to regulate adequate NKT cell responses.

A model for self-control of NKT cell activity is proposed: naïve NKT cells express pro-apoptotic genes to prevent activation and increased autoreactivity in the absence of infection or inflammation. At this stage, NKT cells also express cytokine and chemokine receptors for rapid activation. Upon activation by their cognate antigens (self or non-self) NKT cells produce anti-apoptotic and apoptosis regulating genes for prolonged survival and appropriate inactivation after stimulation. This provides the opportunity to perform their various effector and regulatory function as implied by upregulation of various cytokines and chemokines after activation.

The appearance of apoptosis and cell cycle control genes shows that proliferation and cell responses after activation are tightly controlled. This ensures that NKT cell responses are adequate and rapidly turned off. NKT cells respond rapidly and vigorously to foreign antigen stimulation produce IFNγ and IL-4 after stimulation (Smyth and Godfrey, 2000). Moreover, it is obvious that NKT cells produce a much greater amount of different cytokines after stimulation, which influence other cells of the immune system and helps to coordinate appropriate immune responses.

6.2 NKT cell activity: induction by iGb3 is controlled through antigen availability

Lysosomal storage disorders are a family of over 40 distinct genetic diseases, each of which is a specific defect in lysosomal function. These defects lead to the accumulation of compounds, which are normally degraded, within lysosomes. Lysosomal storage disorders can be classified broadly by the type of material that is accumulated (e.g. lipid storage disorders, mucopolysaccharidoses, glycoproteinoses), although many of these conditions show significant similarities in pathological outcome. Morbus Gaucher and Fabry disease are the most prevalent forms of lysosomal storage disorders (Brady, 1982). Morbus Gaucher, the most frequent lysosomal storage disorder, is caused by a defective lysosomal enzyme, acid-β-glucosidase (GlcCerase), leading to glucosylceramide (GlcCer) accumulation, particularly in cells of the macrophage lineage.

Fabry disease, also referred to as Anderson-Fabry disease, is an X-chromosome-linked recessive lysosomal storage disorder caused by a defective lysosomal αGalactosidase A (αGalA, also termed ceramide trihexosidase). αGalA is the lysosomal enzyme responsible for the breakdown of globotriaosylceramide (Gb3) and related glycosphingolipids (Desnick and Brady, 2004). These glycosphingolipids have a terminal α-galactosyl residue and are components of most cell membranes. Gb3 is normally broken down by αGalA to galactose and lactosylceramide. The deficiency of αGalA leads to progressive accumulation of Gb3 within the lysosomes of most cells types in the plasma. If untreated, Fabry disease is characterized by renal, cardiac, and cerebrovascular manifestations and premature death. A milder phenotype occurs in patients with reduced but detectable αGalA activity. Severe forms of Fabry disease predominantly affect males, whereas female (heterozygous) carriers show mild to severe degrees of the disease. This is caused by random X-chromosomal inactivation (Desnick et al., 2003).

In vivo relevance of iGb3

Zhou et al described iso-globotrihexosylceramide (iGb3) as the endogenous NKT cell antigen. Absence of iGb3 blocks NKT cell development (Zhou et al., 2004). Gb3 and iGb3 differ only in the position of the terminal galactose. Gb3 displays a 1→4 attachment and a 1→3 linkage is found in iGb3. Although a minor structural difference, Gb3 does not activate NKT cells (Figure 16). Modifications of NKT cell antigens were previously shown to have a major impact on successful presentation by CD1d (Kawano et al., 1997). Zhou et al showed that β-hexosaminidase $b^{-/-}$ ($Hexb^{-/-}$) mice have decreased NKT cell numbers due to a lysosomal defect in iGb3 generation from iGb4 by β-hexosaminidase A and B (Sango et al.,

1996). Moreover, iGb3 stimulates NKT cells in a similar manner as αGalCer. Synthesis of iGb3 from lactosylceramide by lactosylceramide-4-α-galactosyltransferase (iGb3 synthase) leads to iGb3 synthesis in the Golgi apparatus. However, iGb3 degradation and loading of lipids onto CD1d occurs in lysosomes (Figure 33). It needs to be further studied to which extend iGb3 is available for CD1d loading and presentation in HexB$^{-/-}$ mice.

The report by Zhou et al did not address regulation of NKT cell activation in normal mice, where iGb3 presence could lead to autoreactivity. iGb4 is also not the only substrate of HexB, with disialoganglisoside (GM2), asialoGM2 (GA2) and globotetrahexosylceramide (Gb4) as further HexB substrates (Sandhoff and Christomanou, 1979). Deficiency in β-hexosaminidase A and B leads also to Gb4 accumulation, a characteristic of Sandhoff disease, whereas deficiency in β-hexosaminidase A only, leads to GM2 accumulation known as Tay-Sachs disease (Platt et al., 2003). Previously, other endogenouse lipids were described as lipid antigens presented by CD1d. Disialoganglioside (GD3), a ganglioside expressed on human melanoma, is able to elicit a NKT cell response. However, GD3 is only present in human melanoma cells and NKT cell activation depends on cross-presentation by professional APCs (Wu et al., 2003).

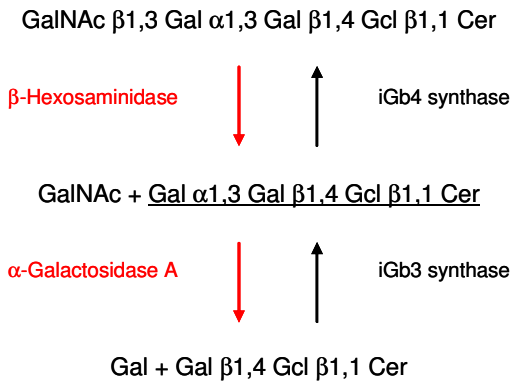


Figure **33**: Schematic of iGb3 synthesis in the golgi (black arrows) and degradation in lysosomes (red arrows). From top to bottom, iGb4, iGb3 and Lactosylceramide. Gb3 is synthesized and degraded the same way.

iGb3 is common in mammalian organisms and ubiquitously present under normal physiological conditions (Kuehn et al., 1992). This raises the question of regulation of antigen availability and NKT cell activation control. Brigl et al showed that NKT cell activation can be achieved by two different pathways: direct TCR mediated activation of NKT cells by ligands presented by CD1d or TLR mediated APC-activation and IL-12 production in combination with recognition of endogenous self lipids by NKT cells. APC-derived IL-12 is important for NKT cell activation in the latter scenario (Brigl et al., 2003). The *in vivo* relevance of iGb3 as endogenous CD1d ligand was demonstrated by failure of HexB$^{-/-}$ mice to activate NKT cells during salmonella infection (Mattner et al., 2005). In contrast, NKT cells in HexB$^{-/-}$ mice responded normally to infection with sphingomonas and lipids thereof. Salmonella and sphingomonas are both gram^{-} bacteria but sphingomonas is LPS-negative whereas LPS is present in the cell wall of salmonella. Furthermore, sphingomonas is an infrequent pathogen with low virulence (Hsueh et al., 1998). Salmonella LPS activates APCs via TLR4 whereas

sphingomonas lipids are directly recognized by the NKT cell receptor via CD1d. Moreover, *Griffonia simplicifolia* I-B4 (IB4), a lectin binding terminal α-galactose, inhibited recognition of iGb3 by NKT cells (Kinjo et al., 2005; Mattner et al., 2005). Taken together, dependency on an endogenous NKT cell antigen / CD1d ligand during TLR-mediated activation was demonstrated.

Regulation of NKT cell activation during lysosomal storage diseases

The critical question is how NKT cell activation is regulated since ubiquitous presence of iGb3 would cause continous NKT cell stimulation. Activation induced cell death could lead to NKT cell depletion. Under physiological conditions, Gb3 and iGb3 are rapidly degraded by lysosomal αGalA, probably reducing antigen availability. In case of defects in enzymatic degradation antigens accumulate and surmount the concentration threshold for effective CD1d loading and NKT cell stimulation. Cells from Fabry patients, deficient for αGalA contain increased levels of Gb3 and probably iGb3.

CD40L treatment of immature DCs induces maturation and enhanced antigen presentation (Fujii et al., 2004). CD40L matured DCs from Fabry patients, stimulated NKT cells indicating that availability of endogenous antigens on the cell surface is below threshold in unstimulated APCs and increased in mature DCs (Figure 17). Lack of NKT cell activation even upon maturation of DCs from healthy individuals, pinpoint antigen availability as an important regulator for NKT cell activation. Surprisingly, CD40L matured DCs from Fabry patients failed to induce a NKT cell response upon exogenous loading of iGb3. It was speculated that the concentration of added antigen was cytotoxic. However, DCs from healthy donors loaded with the same amount of iGb3 stimulated NKT cells. Deficiency of lysosomal lipid degradation resulting in massive lipid accumulation within the endosomal system could influence the antigen processing and presentation machinery. CD1d molecules bind their cognate antigen in the endosomal system. Congestion or deviation of this system could hinder CD1d-mediated NKT cell activation.

Investigations about the effect of Fabry disease on the immune system are very limited. Immune-deficiencies or differences in immune responses in Fabry patients compared to healthy individuals have not been reported. Examination of CD1d-expression by DCs from Fabry patients did not reveal any differences to that of DCs from healthy donors (data not shown). Lack of differences in NKT cell numbers of Fabry patients and healthy individuals further indicate that such a condition does not influence the immune status. It further shows that accumulation of Gb3 and iGb3 does not alter the NKT cell compartment.

Effects of IL-12 and IL-15 on NKT cell activation by iGb3

It has been shown that APC derived IL-12 can bias the self-reactive NKT cell response toward IFNγ and induce NKT cell responses after antigen recognition by TLRs (Brigl et al., 2003; Vincent et al., 2002). IL-15 is important in the development and expansion NKT cells (Matsuda et al., 2002). However, various concentrations of IL-12 or IL-15 did not effect NKT cell responses (data not shown). Treatment of DCs from Fabry patients or healthy donors with IL-12 or IL-15 in the presence or absence of iGB3 did not induce NKT cells activation. This was unexpected considering reports showing IL-12 induced NKT cell activation and its lack in the absence of iGb3 (Brigl et al., 2003; Mattner et al., 2005). Additional factors such as co-stimulatory molecules / receptors could be important for effective NKT cell induction.

iGb3 / iGb3 analogues and regulation of antigen availability

iGB3 analogues were chemically synthesized to analyse the influence of modifications on lipid or sugar moiety of antigens for NKT cell activation. Moreover, they were used to examine the uniqueness of iGb3 as the only endogenous NKT cell antigen and to investigate the specificity of iGb3 recognition by NKT cells. Changes within sugar or lipid residues of CD1d ligands drastically change recognition by NKT cells (Fischer et al., 2004; Kawano et al., 1997).

For instance, the presence and configuration of double bonds within the acyl chain were important for CD1d binding whereas a higher degree of unsaturation led to a stronger NKT cell response (Rauch et al., 2003). Moreover, Sakai et al showed that βGalCer does not induce NKT cell responses stressing the importance of an α-configuration for the terminal sugar (Sakai et al., 1999). PW15 and PW17 differed only slightly from iGb3 as depicted in figure 18. PW15 possesses a saturated acyl chain whereas iGb3 displays a double bond in the respective acyl chain. PW17 is a chimera of the iGb3 sugar moiety and the αGalCer lipid moiety. Because PW17 comprises the αGalCer lipid part and the iGb3 sugar moiety it should be the ideal antigen for NKT cells. Removal of the terminal galactose of PW16 and PW18 by β-galactosidase results in αGlcCer and βGlcCer, respectively (Figure 18). αGlcCer was previously shown to activate NKT cells whereas βGlcCer fails to elicit a NKT cell response (Kawano et al., 1997). This observation was confirmed by strong cytokine induction by PW16. However, PW18 did also elicit a NKT cell response though weaker compared to PW16 or αGalCer. Moreover, lactosylceramide, which has a double bond instead of a hydroxyl group present in PW18, does not activate NKT cells, which is contradictory to previous observations (Kawano et al., 1997; Sakai et al., 1999). PW17 induced a less vigorous NKT cell response. iGb3 analogues were tested with DCs from healthy donors, containing functional αGalA which rapidly degrades iGb3 and iGb3 analogues PW15 and PW17. Jirimycin, a specific inhibitor of αGalA was used to block αGalA activity in DCs from

healthy donors. This should increase the amount of iGb3 available for antigen presentation and thus lead to NKT cell stimulation. Jirimycin addition increased IFNγ and IL-4 production. In the case of PW17 this strengthens the hypothesis that NKT cell activation is controlled by antigen availability. NKT cell responses to PW18 were slightly elevated in the presence of jirimycin, probably due to elevated b-galactosidase activity in the absence of αGalA (Figure 19). This phenomenon has been observed previously for other enzymes involved in lysosomal degradation (Mayes et al., 1981).

Stimulation of NKT cells by iGb3 and iGb3 analogues through DCs of healthy donors or Fabry patients was subject to considerable variability. This ranged from profound IFNγ and IL-4 production and proliferation to minute or even missing responses. Addition of iGb3 to DCs incubated with αGalCer did not inhibit or influence NKT cell activation demonstrating no inhibitory effect of iGb3. Reasons for this observation should therefore be attributed either to differences concerning APCs or NKT cells. APCs showed normal capacities for antigen processing and NKT cell stimulation as shown by the profound responses induced by PW16. FACS analysis showed normal expressions of DC specific marker such as CD11c and CD1b (data not shown) indicating that the DC phenotype did not contribute to these variations. NKT cells used in these experiments were from a human polyclonal NKT cell line. This cell-line was generated on the basis of cell-morphology and Vα14 TCR expression and was shown to respond to αGalCer. Nevertheless, it is possible that minor differences between NKT cell lines cause these variations.

Activation induced cell death of NKT cells

Despite all the discrepancies mentioned before, iGb3 induced a strong increase in NKT cell numbers in bulk cultures. NKT cell numbers were drastically increased in PBMCs of healthy donors cultured for 4 days in the presence of iGb3 and iGb3 analogues at various concentrations. Strikingly, the opposite was the case when PBMCs from Fabry patients were used (Figure 20). Decreases in NKT cell numbers to minimal levels by higher concentration of antigen could be attributed to activation induced cell death of NKT cells. As described above, antigen availability is crucial to regulate NKT cell activation. Increased presentation of iGb3 on the surface of APCs, even in the absence of infection, could lead to a pre-activated phenotype of NKT cells; a characteristic also revealed by microarray analysis. Addition of iGb3 would lead to increased antigen availability and amplified NKT cell activation. This could finally result in activation induced cell death as revealed by drastically reduced cell numbers. Activation induced cell death is controversially discussed for NKT cells. Leite-de-Moraes et al showed activation induced cell death of NKT cells after stimulation with IL-12 and IL-18 and Fas/FasL interaction (Leite-de-Moraes et al., 2000). Conversely, it was

demonstrated that NKT cells are relatively resistant to activation induced cell death compared to other T cells and rather downregulate their TCR thereby disappearing from detection (Emoto et al., 1999a; Harada et al., 2004). However, analysis of gene expression in activated NKT cells indicates the existence of sophisticated mechanisms to control NKT cell activity and to balance prolonged survival and apoptosis. It remains to be elucidated which factors and conditions influence this balance.

The results suggest that a deficiency in lysosomal processing and degradation, leading to an accumulation of NKT cell ligands, is critically important for regulation of NKT cells. Moreover, continuous presence of iGb3 in the case of enzyme deficiency with additional appearance of NKT cell ligands, causes dramatic loss of NKT cells by activation induced cell death.

6.3 Characterization of PIM-reactive NKT cells

Mycobacterial PIM was identified by our group as a bacterial antigen recognized in a CD1d-restricted fashion by NKT cells (Fischer et al., 2004). None of the other mycobacterial lipids tested showed binding to CD1d, including LAM a lipid antigen for human CD1b (Brigl and Brenner, 2004).

The only CD1d-restricted antigen from a pathogen described until recently was the GPI anchor of proteins derived from parasitic protozoans such as *Plasmodium spec.*, although these data remain controversial (Schofield et al., 1999; Molano et al., 2000; Procopio et al., 2002). Intact PIM is recognized by T cells in a CD1d-restricted way (Figure 21). The high frequency of αGalCer-CD1d tetramer$^+$ T cells, compared with the lower frequency of PIM-CD1d tetramer$^+$ T cells, could be explained by assuming that PIM-reactive NKT cells constitute a distinct subpopulation of NKT cells within the αGalCer-reactive NKT cells. This hypothesis was supported by findings that both NKT cell populations predominantly expressed the Vβ8 TCR chain together with the invariant TCR α-chain. Infection of mice with *M. bovis* BCG led to a decrease in detection of reactive NKT cells confirming a previous report (Emoto et al., 1999a) (Figure 25). The frequency of PIM-reactive NKT cells, although with lower cells number followed a similar kinetic upon *M. bovis* BCG infection. However, this indicates further that PIM-reactive NKT cells are functionally and structurally similar to αGalCer-reactive NKT cells. The difference in reactivity to αGalCer or PIM could be explained by fine differences in the sequence of the TCR. The low frequency of PIM-reactive NKT cells did not allow purification of sufficient numbers of PIM-reactive NKT cells for subsequent sequence analysis. Decreased NKT cell numbers during infection is controversial. Previously, it was published that NKT cells undergo apoptosis after stimulation

(Emoto et al., 1999a; Leite-de-Moraes et al., 2000; Osman et al., 2000). Recent data showed that NKT cells rather downregulate their TCR and surface markers after activation and are therefore not detectable by CD1d tetramer staining (Crowe et al., 2003; Matsuda et al., 2000; Seino et al., 2004). It could be hypothesized that both scenarios occur, i.e. some NKT cells downregulate their TCR while others undergo apoptosis after stimulation. The slight increase in cell numbers of PIM-reactive, tetramer$^+$ but NK1.1-negative NKT cells indicate that some NKT cells could disappear from detection by downregulation of their surface markers (Figure 25). However, this may not explain the marked loss in the overall number of NKT cells after infection.

Apart from αGalCer, none of the tested lipids, including PIM, induced a notable IL-4 response in NKT cells. αGalCer analogues, selectively inducing IL-4, but not IFNγ, suggest that distinct moieties of the glycolipid antigens determine the cytokine pattern; probably by modulation of APCs (Miyamoto et al., 2001). Since PIM displays less antigenic potency than αGalCer, the lack of IL-4 induction by PIM could suggest a higher signalling threshold for IL-4. The potency of an antigen would thereby determine the type and the vigorousness of an immune response. IFNγ is a key cytokine during mycobacterial infections. It promotes Th1 cell development and leads to macrophage activation. Thus, recognition of mycobacterial PIM, presentation by CD1d and subsequent activation of NKT cells could play a role in the immune response during infection with *Mycobacterium tuberculosis* (Szalay et al., 1999). The fact that mice, deficient for CD1d are equally susceptible to a high-dose i.v. inoculum of *M. tuberculosis* as are wild-type B6 mice (Behar et al., 1999) does not exclude a conditional role for CD1d-restricted T cells in infection. This is supported by a report showing that homozygous, Jα281$^{-/-}$ mice, devoid of NKT cells, appear to be more susceptible than heterozygous mice, at least in later stages of tuberculosis (Sugawara et al., 2002). Furthermore, glycosphingolipids and sulfatide variants of sphingomonas were recently shown to activate NKT cells in a CD1d-dependent manner and contribute to immune responses and survival of mice infected with these bacteria (Kinjo et al., 2005; Mattner et al., 2005; Wu et al., 2005). Yet, sphingomonas is an infrequent pathogen with low virulence (Hsueh et al., 1998).

These results demonstrate capacities for CD1d-restricted T cells ranging from autoimmunity and immune regulation to antimicrobial host defence, by recognition of bacterial lipid antigens and subsequent IFNγ production.

6.4 Toll like receptor vs. T cell receptor mediated recognition of mycobacterial lipids

CD1d-mediated NKT cell activation involves antigen presentation and specific recognition of the respective antigen through the TCR. Like MHC class I presentation, CD1d-mediated antigen presentation is part of the adaptive immune response. It is antigen specific and requires antigen processing and presentation by professional APCs (Pan et al., 1999; Prigozy et al., 2001). Recognition of pathogenic microbes by the innate immune system is facilitated by TLRs. TLRs recognize pathogen associated molecular patterns (PAMPs) which are highly conserved structures found in many bacteria, parasites and viruses (Medzhitov, 2001). TLR-mediated immune activation constitutes a major defence mechanism of innate immunity. Innate and adaptive immunity are highly interwoven with each other and bacteria elicit both innate and adaptive immune responses, yet at different timepoints (Michalek et al., 2002; Smith et al., 2005). NKT cells have a preactivated phenotype and respond vigorously to antigenic stimulation. Additionally, they are thought to play an immuno-regulatory role at the interface of innate and adaptive immunity (Kronenberg and Gapin, 2002).

TLR2 activation of mycobacterial lipids

Whole lipid extracts as well as crude PIM fractions elicited a clear NF-κB activation in a dose dependent manner which depends on recognition by TLR2. In contrast, PI and lyso-PIM, which are structurally similar to PIM, were not recognized by TLR2 (Figure 26). Notably, removal of one acyl chain from PIM by phospolipase A creates lyso-PIM and results in loss of TRL2 activation. PI represents the core structure of various mammalian cell membrane lipids and GPI anchored proteins and did not activate TLR2. GPIs consist of a conserved glycan structure, ethanolamine-phosphate-6Manα1-2Manα1-6Manα1-4GlcNα(1-6)-linked to PI. Their function is to anchor certain cell surface proteins in distinct membrane domains (Englund, 1993). PI and GPI are conserved throughout evolution, although exhibiting significant biosynthetic differences between mammals and microbes (Tiede et al., 1999). Bacterial GPI is a direct target of the innate immune system inducing pro-inflammatory responses (Ropert and Gazzinelli, 2000). Additionally, PI constitutes the core component of PIM, but lacks the sugar moiety composed of mannose residues (Brennan and Besra, 1997). Nevertheless, lack of NF-κB induction by PI was expected, since it is part of host cell structures (Villeneuve et al., 2005). In line with previous publications, lack of NF-κB induction by PI and lyso-PIM confirms that distinct bacterial structures differ in their biological activity depending on affinity to TLRs (Moreillon and Majcherczyk, 2003; Morr et al., 2002). TLR2 recognition of PI would result in constant induction of immune responses against endogenous structures, leading to autoreactivity.

These results emphasize the importance of discrimination between conserved mammalian and bacterial structures to evoke a TLR mediate innate immune response.

Dual antigenic potential of mycobacterial lipids

It has been demonstrated previously that mycobacterial PIMs are recognized by TLR2 and activate NKT cells in a TCR independent manner (Gilleron et al., 2001; Gilleron et al., 2003). Analysis of fractions isolated from crude PIM extracts detected fraction 7 to elicit CD1d-dependent NKT cell activation as well as TLR2-mediated NF-κB induction (Figures 29 and 30). None of the other lipid fractions induced NKT cells in a CD1d-dependent manner, but were recognized by TLR2. This indicates that only fraction 7 contains a compound capable of specific TCR-mediated as well as non-specific activation of immunity. However, structural analysis of the different fractions by MALDI-MS did not determine this compound unequivocally. In fraction 16, predominantly PIM_6Ac_3 and PIM_6Ac_4 were detected as the major compounds. Minor peaks indicate the presence of other, minor compounds. Along with PIM_6Ac_2, PIM_6Ac_3 and PIM_6Ac_4 were also detected in fractions 19 and 21. Strikingly, PIM_6Ac_3, PIM_6Ac_4 and PIM_6Ac_2 were absent from fraction 7. MALDI-MS of fraction 7 revealed a cluster of peaks from the range of 1233.97 to 1640.51 Da (Figure 31). These peaks appear only when measured in positive mode MALDI-MS, indicating that they are not negatively charged. Moreover, masses (m/z) indicate that these compounds have a low number of mannoside residues. Furthermore, all peaks are distant from each other by 14 mass units which is representative for CH_2 groups. Therefore, compounds detected in fraction 7 contain fatty acids of different length. This indicates that the active compound in fraction 7 could be a low mannosylated PIM. Conversely, some of the peaks found in fraction 7 were detected in fraction 16 and 21. Within these fractions they were only very minor compounds, whereas they constitute the only peaks present in fraction 7. It was shown that, enriched low mannosylated PIM_2 recruit NKT cells upon s.c. injection, but in a TCR-independent manner (Gilleron et al., 2001; Mempel et al., 2002). Additionally, semipurified PIM_2 fractions failed to trigger selective expansion of both human and murine NKT cells (Fischer et al., 2004). Moura et al showed that mycobacterial lipids inhibit T cell activation (Moura and Mariano, 1997). In line with this observation our findings could indicate that lipids present in fraction 16, 19 and 21 but absent in fraction 7 can suppress activation of NKT cells. Further purification of the different fractions and analysis by NMR and GC-MS will allow identification of these active lipids within these fractions to attribute the distinct activating properties.

Direct activation of NKT cells by TLRs expressed on their cell surface could also attribute to immune responses to mycobacteria. mRNA expression of TLR2 in NKT cells was previously described (Shimizu et al., 2002). However, this could not be verified by FACS analysis for

surface and/or intracellular expression of TLR2 and TLR4 on NKT cells. Therefore, direct activation of NKT cell-expressed TLR2 and TLR4 can be excluded.

In summary, different PIMs released by mycobacteria may be responsible for attraction of NKT cells to the site of infection in a TCR independent way and further activation can occur through a TCR-dependent activation step of NKT cell population.

A model of NKT cell activation by mycobacterial lipids

A three step model for NKT cell activation can be proposed:

1) TCR independent NKT cell activation
   NKT cells are recruited in a TCR independent manner to sites of infections and play a vital role in the formation of granulomas. This recruitment was shown to be associated with the presence of mycobacterial PIMs but irrespective of the presence of CD1d (Apostolou et al., 1999; Gilleron et al., 2001). The underlying mechanism remains to be identified.

2) APC / TLR / IL-12 mediated, TCR dependent NKT cell activation
   TLRs expressed on professional APCs recognize bacterial PAMPS and lead to activation of the APCs resulting in IL-12 production. Endogenous ligands presented by CD1d are specifically recognized by the NKT cell TCR. This results in a pre-activation of NKT cells. NKT cells are fully activated by APC derived IL-12 in combination with TCR dependent recognition of endogenous lipids. Missing presentation of endogenous ligands abolishes NKT cell activation.

3) CD1d mediated and antigen specific TCR dependent NKT cell activation
   Bacterial lipid antigens are taken up and processed by professional APCs. Subsequently these antigens are loaded onto CD1d in lysosomes and presented on the cell surface. CD1d presented antigens are specifically recognized by the invariant TCR and yield in NKT cell activation as shown for lipid antigens from mycobacteria, sphingomonas and ehrlichia.

Combined presence of these pathways highlight the antimicrobial NKT cell functions by surveying both endogenous and bacterial glycosphingolipids by a TCR dependent manner, as well as being recruited to sites of infection in a TCR independent manner. Supportive of this, transcriptome analysis demonstrate the vast capacities of NKT cells to respond to various stimuli and to perform a variety of effector and regulatory functions during immune responses.

6.5 Towards an understanding of NKT cells

A global picture on NKT cell properties and functions as well as regulation of NKT cell activity could be revealed. NKT cells show an early response to infection mediated by rapid activation and characterized by constitution of a wide arsenal of effector and immuno-regulatory functions. They can be non-specifically induced by inflammation and are important for establishing granuloma-like lesions. This activation was demonstrated to be a TCR independent process of NKT cell recruitment to sites of infection. Furthermore, activation of NKT cells by bacterial antigens can be either direct, TCR mediated by ligands presented by CD1d. Alternatively, TLR mediated APC-activation leads to IL-12 production which activates NKT cells recognizing their endogenous lipid antigen presented by CD1d. Activation of NKT cells via the recognition of endogenous antigens is critically dependent on antigen availability.

NKT cells are by definition self-reactive displaying a pre-activated status which is tightly controlled. Upon activation, effector functions of NKT cells are initiated as revealed by increased expression of various cytokines and chemokines. This enables NKT cells to competently regulate and modulate immune responses. Both, naïve and activated NKT cells display elevated expression of apoptosis regulating genes providing NKT cells with high degree of self-control to precisely regulate their own activity.

7 Summary

NKT cells are involved in the regulation of a wide range of immune responses. They play a role in autoimmunity, antitumour responses, host defence to infections and in allergy and inflammation. In contrast to classical T cells, NKT cells respond to lipid antigens and rapidly produce IFNγ and IL-4 as well as other cytokines with immuno-regulatory capacities. Although various publications describe NKT cells in infections, inflammation or antitumour immunity there is only very limited information about the mechanism of NKT cell activation. Moreover, natural antigens were not known until recently.

1) Microarray analysis comparing NKT cells to NK cells, conventional $CD4^+$ T cells and regulatory T (Treg) cells were designed to examine a global gene expression profile of NKT cells in the absence or presence of an exogenous stimulus. The profiles revealed a NKT cell specific gene expression pattern as well as expression of genes which NKT cells share with NK cells, conventional $CD4^+$ T cells and Treg cells.
   The latter ones allow the distinction between those genes which are rather attributed to innate responses, i.e. shared with NK cells or those of the T cell-effector / -regulatory phase, i.e. shared with effector T cells or Treg cells. This suggests broad functional capacities of NKT cells.
   The group of genes, uniquely regulated in NKT cells codes for cytokine and chemokine receptors, regulators of cell proliferation / activation and proteins involved in cell migration and cytokine processing. This profile demonstrates capability of NKT cells for vigorous reaction to various stimuli, rapid response by cytokine and chemokine production and strict self-control of activity.
   Genes with similar expression levels of NKT cells and NK cells, conventional $CD4^+$ T cells or Treg cells expanded the knowledge of innate immunity-like or adaptive immunity-like properties of NKT cells. Detection of additional cytokines, chemokines, cytokine/chemokine receptors, as well as granzyme B or FasL emphasizes regulatory and effector functions of NKT cells.
   Genes induced upon αGalCer mediated activation of NKT cells consisted almost exclusively of cytokines, chemokines and proliferation control genes. Among these genes were IFNγ, IL-4, TNFα, IL-17, lymphotactin, Gadd45/Myd118 and Map_3K_8. This collection of genes proves the profound ability of NKT cells to modulate immune responses by prolonged and substantial production of cytokines and chemokines.

2) Microarray analysis increased the insight into the genetic profile of NKT cells and revealed gene expression patterns of activated NKT cells. However, regulation of NKT

cell activation by endogenous and exogenous antigens still remains to be determined. Regulation of NKT cell activation was examined by employing the endogenous NKT cell ligand iGb3 as well as PBMCs from patients with Fabry disease. Fabry patients have a genetic defect for the lysosomal αGalA and display an extensive sphingolipidosis. Comparison of normal cells and those from patients with Fabry disease revealed that regulation of antigen availability plays a crucial role in regulation of NKT cell activation. Moreover, uncontrolled appearance and increased concentrations of the endogenous antigen iGb3 led to substantial decrease in NKT cell number, presumably by activation induced cell death.

3) In addition to the endogenous ligand iGb3, NKT cells recognize bacterial lipid antigens. A preceding study from this group showed that mycobacterial PIM is recognized by NKT cells in a CD1d specific, TCR mediated fashion. We could show that PIM are recognized by human and murine NKT cells and induce IFNγ and IL-4 production. Subsequent experiments to characterize PIM-reactive NKT cells were performed in this thesis. FACS analysis and infection studies with *M. bovis* BCG showed that PIM-reactive NKT cells are a small subset within αGalCer-reactive NKT cells specifically recognizing mycobacterial lipids.

4) Mycobacterial lipids were furthermore analyzed for their capacity to elicit TLR2-mediated immune responses in addition to or substitution for an NKT cell response. It was demonstrated that different lipid fractions isolated from *M. bovis* BCG were recognized by TLR2 and elicited a potent NF-κB activation. One of these fractions also induced a CD1d-mediated NKT cell response. So far, structural analysis by MALDI-MS of the different fraction did not help to identify the antigen with dual capacity for specific TCR- and unspecific TLR- mediated immune activation. Further purification and analysis of NKT cell activation will help to determine this compound. This will also determine the structural requirements for TLR and TCR recognition.

NKT cells could be described as immuno-regulatory, self-limiting cells additionally equipped with effector functions. This décor represents the fundamental basis for their ability to modulate diverse immune responses. The existence of multiple activation mechanisms by bacterial antigens enables NKT cells to efficiently monitor the immune system and participate either in a broad, unspecific or pathogen/antigen specific fashion. NKT cell subsets could be vital for the recognition of distinct pathogens as demonstrated for mycobacteria, sphingomonas and ehrlichia.

8 Zusammenfassung

NKT-Zellen sind an der Regulation einer Vielzahl von Immunantworten beteiligt. Sie spielen eine Rolle in Autoimmunität, Tumorerkennung, Schutz gegen Infektionen und in allergischen und entzündlichen Prozessen. NKT-Zellen erkennen, im Vergleich zu klassischen T Zellen, nicht-körpereigene Glykolipidantigene und produzieren eine Vielfalt von Zytokinen mit immuno-regulatorischen Eigenschaften sehr schnell nach Aktivierung. Darüber hinaus besitzen NKT-Zellen die einzigartige Fähigkeit IFNγ und IL-4 gleichzeitig zu produzieren. Obwohl Funktionen von NKT-Zellen in diversen Immunantworten beschrieben wurde, gibt es nur sehr limitiert Informationen über die Mechanismen der Aktivierung von NKT-Zellen. Zudem waren bis vor kurzem keine natürlichen NKT-Zell-Antigene bekannt.

1) Microarray Analysen wurden durchgeführt um ein Genexpressionsprofil von NKT-Zellen zu erhalten. Dazu wurden NKT-Zellen mit NK-Zellen, konventionellen $CD4^+$ T-Zellen und regulatorischen T-Zellen (Treg) sowie in An- oder Abwesenheit eines exogenes Stimulus untersucht. Es konnte ein NKT-Zell-spezifisches Genexpressionsprofil erstellt werden. Darüber hinaus zeigte sich, dass sich die Expression bestimmter Gene in NKT-Zellen und NK-Zellen, konventionellen $CD4^+$ T-Zellen oder Treg-Zellen gleicht und Eigenschaften sowohl der angeboren als auch der adaptiven Immunantwort NKT-Zellen zugeordnet werden können. Expression von Genen die Zytokine und Chemokine, Zytokin/Chemokin Rezeptoren aber auch Granzyme B oder FasL codieren, betonen die regulatorischen und Effektorfunktionen von NKT-Zellen und zeigen die vielfältigen funktionellen Kapazitäten von NKT-Zellen auf.

   NKT-Zell-spezifische Expression konnte für eine Reihe von Genen, involviert in Regulation der Zellproliferation / Aktivierung, Zellmigration, Prozessierung von Zytokinen und für Zytokin- und Chemokin-Rezeptoren, nachgewiesen werden. Dies demonstriert die Fähigkeit von NKT-Zellen zur raschen Antwort auf verschiedene Stimuli, schnellen Produktion von Zytokinen und Chemokinen und verdeutlicht eine strikte Selbstkontrolle der Aktivität.

   In αGalCer-aktivierten NKT-Zellen konnten fast ausschließlich Gene für Zytokine, Chemokine und zur Proliferationskontrolle detektiert werden. Unter diesen Genen waren: IFNγ, IL-4, TNFα, IL-17, lymphotactin, Gadd45/Myd118 and Map_3K_8. Das Expressionsprofil aktivierter NKT-Zellen belegt die ausgeprägte Regulation der Aktivität und die Kapazität zur vielfältigen Modulation von Immunantworten durch extensive Zytokin- und Chemokin-Produktion.

2) Mit Hilfe der Transkriptome Analyse konnte das genetische Profil von NKT-Zellen und das Expressionsmuster nach NKT-Zell-Aktivierung demonstriert werden. Die Regulation der NKT-Zell-Aktivierung durch endogene und exogene Antigene jedoch nicht geklärt werden. Der endogene NKT-Zell-Liganden iGb3 und Lymphozyten von Patienten mit der Fabry Krankheit wurden benutzt um die Regulation der NKT-Zell-Aktivierung zu untersuchen. Fabry Patienten haben einen genetischen Defekt für das lysosomale Enzym αGalA und entwickeln eine ausgeprägte Sphingolipidose. Vergleiche von normalen Zellen und Zellen von Fabry Patienten zeigten, dass die Regulation der Antigenverfügbarkeit ausschlaggebend für die Regulation der NKT-Zell-Aktivierung ist. Darüber hinaus konnte gezeigt werden, dass unkontrolliertes Vorhandensein und erhöhte Konzentration des endogenen Liganden iGb3, zu einer erheblichen Verringerung der NKT-Zell-Zahl, vermutlich durch Aktivierungs-induzierten Zelltod, führt.

3) NKT-Zellen erkennen neben iGb3 auch bakterielle Lipidantigene. Eine frühere Arbeit dieser Gruppe konnte zeigen, dass mycobakterielles PIM durch den T-Zell-Rezeptor (TZR) der NKT-Zellen, in CD1d-abhängiger Weise erkannt wird. Wir konnten zeigen, dass PIM von humanen und murinen NKT-Zellen erkannt wird und die Produktion von IFNγ und IL-4 induziert. Die Charakterisierung der PIM-reaktiven NKT-Zellen wurden im Rahmen dieser Arbeit durchgeführt. FACS Analyse und Infektionsstudien mit *M. bovis* BCG zeigten, dass PIM-reaktive NKT-Zellen eine Subpopulation innerhalb der αGalCer-reaktiven NKT-Zellen sind.

4) Mycobakterielle Lipide wurden weiterhin auf ihre Fähigkeit untersucht, eine TLR2-vermittelte Immunantwort, zusätzlich oder anstelle einer NKT Zellaktivierung, hervorzurufen. Es wurde gezeigt, dass verschiedene Lipidfraktionen von *M. bovis* BCG von TLR2 erkannt werden und zur Aktivierung von NF-κB führen. Eine dieser Fraktionen induzierte darüber hinaus auch eine CD1d-vermittelte NKT-Zell-Antwort. MALDI-MS-Analyse der verschiedenen Fraktionen konnte das Antigen mit Potential zur unspezifischen TLR2- und spezifischen NKT-Zell-Aktivierung allerdings nicht eindeutig identifizieren. Weitere Aufreinigungen und Analyse der NKT-Zell-Aktivierung werden helfen diese Substanz zu identifizieren und die strukturellen Anforderungen für die Erkennung von Antigenen durch TLR2 und TZR aufzuklären.

NKT-Zellen konnten als immunregulatorische, selbst-limitierende, zusätzlich mit Effektor-Funktionen ausgestattete, Zellen beschrieben werden. Diese Ausstattung bildet die elementare Basis für die Fähigkeit der NKT-Zellen verschiedenste Immunantworten zu modulieren. Die Existenz mehrerer NKT-Zell-Aktivierungsmechanismen durch bakterielle

Antigene befähigt zur effizienten Beobachtung des Immunsystems und zur Teilnahme an Immunantworten, entweder in einer unspezifischen- oder Antigen/Pathogen-spezifischen Art und Weise. NKT-Zell-Subpopulationen könnten für die Erkennung bestimmter Pathogene, wie *Mycobakterium sp., Sphingomonas sp.* and *Ehrlichia sp.*, unerlässlich sein.

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Erklärung

Hiermit erkläre ich, dass ich die vorliegende Doktorarbeit selbständig und nur unter Zuhilfenahme der genannten Literatur und der erwähnten Hilfsmittel angefertigt habe.

Berlin, den Juli 2005

Marcus Niemeyer

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